

nature

August 26, 1976

Which way now for journals ?

BARNABY RICH said in 1613 that: "One of the diseases of this age is the multiplicity of books; they doth so overcharge the world that it is not able to digest the abundance of idle matter that is every day hatched and brought forth into the world". He would undoubtedly have used similar words in 1976 to articulate the concern that most practising scientists express from time to time about the proliferation of journals and the number of papers published, but perhaps he would also have come up with some practical suggestions about how the problem should be solved in the light of diminishing library budgets. It is really only when a university library, say, cuts out by mistake what a substantial number of people regard as a vital journal that there is any significant outcry.

Interestingly, though, only about 55% of the expenditure of a typical university library on journals (in the UK at least) is devoted to 'essential' titles. The remainder are 'desirable' or 'marginal', spending on these categories being 40% and 5% of the total, respectively. If the coming academic year proves to be another tough one financially, marginal titles are likely to become a thing of the past, and the list of desirable ones will be on the chopping block. So if things don't get any better for libraries, what is to be done?

First of all, one might wish for an unwritten rule that the creation of a new journal had to be a true reflection of the appearance of a new subject grouping or synthesis of subjects, and not merely a rather meaningless subdivision of an old subject. (No marks out of 10 for *Slide Rules in Laryngology*, for example.) And on this basis, why not aim to have two journals shut down for every one started? (True, this would imply the publication of fewer papers, but that would be no bad thing in the light of claims that more than 90% of papers written eventually get published somewhere.) The plain facts of the matter, however, are that of about 35,000 journals in existence at present, only 1,300 disappear each year whereas a full 2,000 are added.

Second, as the pious hopes of the previous paragraph can at best be only partly realised, what can be done to existing journals to reduce their bulk and their cost? The bulk is particularly important both because it determines how much the reader has to read, and because postage is now such an important element (some 15% of the cost of a subscription to *Nature* in the UK, for example).

One of the ultimate solutions is to publish a journal

solely in microform—probably microfiche, a single piece of photographic sheet film some 10 × 15 centimetres containing reduced images of 100-odd journal pages. There are no paper or machining charges and minimum postal costs—but the spectre of user resistance rears its head. How can we browse in a library that uses uninviting gadgetry like a microfiche reader, they ask. It seems that nobody is ready yet for a complete revolution like that, however, and maybe they never will be. Microform for archival use, yes; for current use, probably no.

So what are journal publishers (which include many learned societies, large and small, as well as commercial concerns) to do? The most accurate indicators of current thinking are the experimental publishing projects being carried out by the Chemical Society in the UK and the American Chemical Society in the United States. These cautious steps forward involve what are called 'synopsis journals', in which the author writes a synopsis of perhaps 1,000 words, which is printed conventionally and backed up by more detailed information, on microfiche for example, to which the reader can have access if he requires.

As the microfiche part of such a journal can make use of directly photographed typewritten pages while the synopsis part can still be printed in a way that makes it look no different to a comparable publication, the average reader, who likes his journal to look 'right' and to be printed on good old-fashioned paper, can be tempted to move in the right direction without sacrificing any of the things he holds dear. The librarian, for his part, would hope to see the changes reflected in the subscription price.

The browsing reader can still browse in the time-honoured way, with the added advantage of not having so much to read or skim through. Those who need to consult the back-up information will only have to suffer microform in pursuit of the small number of papers that directly interest them and ought not to find its use an excessive chore in these circumstances. In fact, if one believes the tales that most papers are only read in detail by one or two people, it could turn out that librarians in a synopsis era would find relatively little call on their microfiche facilities for this purpose. Which raises the whole question of whether all that detail actually needs to be published at all. Could it not equally well be submitted for scrutiny by the referee and then made available by the author to those who care to request it?



Tennessee valley energy

The Tennessee Valley Authority represents an American historical landmark. **Colin Norman** reports

WHEN the Tennessee Valley Authority (TVA) was established in 1933, at the height of the great depression, it rapidly became one of the more controversial instruments of President Franklin D. Roosevelt's famed New Deal. Designed, in Roosevelt's words, for the reclamation of land and of human beings, the creation of TVA was a unique federal attempt to attack the grinding poverty of an entire region by developing and conserving its natural resources.

Like much of the New Deal, the experiment was controversial because it went against the capitalist grain for the federal government to play such a strong role in economic and social enterprises. To many conservatives, it smacked of socialism. Now, 43 years later, TVA is still controversial, though for different reasons.

In 1933, the Tennessee Valley was an economic and environmental disaster area. Most of the region's people were at the bottom of the pile, living in desperate poverty. The land, which had been worked to death, was fast being eroded and turned into a barren waste. The hillsides, which once boasted the largest hardwood forests in North America, were denuded. And the 1,000-mile Tennessee River would frequently burst its banks, flooding vast areas; in many places, shoals and shallows also made it impossible to navigate. Then along came TVA's armies of engineers, scientists and agriculturalists to dam up the rivers, reforest the hillsides and reclaim the land. By most measures, they have been spectacularly successful.

Although family income in the region is still a little below the national average, it is no longer an economically depressed area. It has a productive agricultural system and industries have been attracted into the area by cheap electricity rates. And the Tennessee River has been tamed by numerous dams, so that it is navigable and it no longer rampages through the fields and towns each spring. The stark before-and-after contrast in the valley in fact gradually made true believers of all but the most diehard conservatives.

Consequently, TVA is now held in the highest regard by many of the people who live in the seven-state area which it serves, a fact painfully discovered by Senator Barry Goldwater in 1964 and by Ronald Reagan a few

months ago. Both hinted that, if elected President, they may consider selling TVA off to private industry to get rid of this socialist monstrosity. They paid dearly at the polls as a result, losing important elections in Tennessee.

Highly charged issues

TVA is controversial now because it is facing many highly charged issues endemic to an industrial society, such as energy planning, strip mining, air pollution, and public participation in governmental decision-making. The sheer scale of TVA's operations ensures that the way it tackles those issues often has national implications.

A description of TVA's activities reads like a list of superlatives. It is the largest energy supplier in the United States, providing electricity to a region the size of Great Britain. It is the single largest user of coal in the nation, consuming more than 40 million tons a year in its power plants. It has more nuclear power plants under construction than any other electric utility. The 33 major dams which have been constructed on the Tennessee River and its tributaries harness lakes whose combined shorelines are longer than those of the Great Lakes. And it is the region's largest employer, providing 31,000 jobs.

Even the criticisms being levelled at TVA are impressive in their scope. During Senate testimony on TVA last year, for example, one critic suggested that "the Tennessee Valley Authority is a part of the problem in Appalachia, not a part of the solution", while another stated that TVA "began as an experiment in democracy but has turned into an example of how our government can practise colonialism on its own people".

TVA is really two separate operations. One, financed entirely by federal appropriations of more than \$100 million a year, is responsible for running a vast range of programmes, including agricultural assistance, fertilizer research and development, and the management of parks, dams and recreational facilities. The other, more controversial, side of TVA's activities is its role as supplier of electricity to the region. It is a self-supporting, \$1,000 million-a-year operation, financed by revenues and by the sale of bonds for capital projects.



Fontana dam, the TVA's highest

Problems, controversies

The problems and controversies encountered by TVA's energy operations are similar to those being faced by other electric utility companies, but TVA's unique structure gives it some advantages in tackling them. For one thing, the agency's Congressional charter requires that it be run as a non-profit enterprise, which means that it doesn't have to pay dividends to stockholders. It is also fortunately situated right next door to the Appalachian coal fields, and its numerous dams provide a secure source of cheap electricity. For those reasons, TVA's electricity is the cheapest in the country, selling at about 60% of the national average.

At present, about 20% of TVA's power comes from hydroelectric sources, and the rest comes from coal-fired power stations. A few years ago, however, the agency decided that all its future energy growth would come from nuclear power plants, and it embarked on a massive nuclear construction programme. By the mid-1980s, TVA expects to have 17 nuclear reactors in operation at seven different sites, adding up to a staggering 20,000 MW. It would be the largest user of nuclear power in the country. Clearly, TVA's espousal of the atom, at a time when many other electric utilities are deferring or cancelling their nuclear orders, has enormous impact on the United States nuclear power programme.

Why does a utility which is sitting on top of a vast coal field decide to go nuclear? TVA has already dammed up most of the rivers in the region so that there is virtually no scope for expanding its hydroelectric generating capacity. "The choice is between coal and nuclear", says TVA General Manager Lynn Seeber, "and we believe

that nuclear will be cheaper and environmentally more acceptable". The agency's critics disagree, however, pointing out that TVA's brief experience so far with nuclear power has been disastrous—it owns and operates the Browns Ferry plant which caught fire last year—and the critics also argue that the cost estimates will prove to be grossly inaccurate.

Be that as it may, environmental factors clearly played a key role in TVA's decision to opt for nuclear power instead of expanding its coal-fired generating capacity. In short, federal pollution control regulations are becoming more and more expensive to meet, particularly since TVA recently lost a major court battle with the Environmental Protection Agency (EPA).

Regulation problems

The pollution control regulations which are causing TVA most of its problems are embodied in the 1970 Clean Air Act, a bill which sets air quality standards and a timetable for meeting them. In some places, the Act is a little fuzzy in its definitions, and there has been a royal battle on several fronts over compliance with the standards. TVA decided early on that it could meet the regulations by a combination of tall smokestacks on its power plants, and continuous meteorological monitoring to ensure that the pollutants are not blown back to the ground in large concentrations.

It developed a sophisticated forecasting and monitoring system which would automatically give orders to curtail, or shut down entirely, individual power plants when meteorological conditions prevent proper dispersal of the stack gases. TVA has recently computerised the entire operation.

EPA interpreted the Clean Air Act differently, however, insisting that it requires stack gases, such as sulphur dioxide, to be removed entirely. Simply spewing them into the atmosphere through tall stacks will cause problems downwind, when the SO_2 is washed out of the atmosphere as so-called acid rain, EPA argued. As usual in such matters, the dispute ended up in the courts and the outcome was closely watched by environmentalists and by industry since it represented a crucial test of EPA's strategies.

TVA lost the battle earlier this year. According to TVA officials, it will cost about \$250 million a year to install and operate the required pollution control equipment on several power plants. By contrast, TVA's proposed meteorological approach would have cost about \$20 million a year.

And there have been other problems with TVA's heavy use of coal. For one

thing, coal prices have escalated from about \$10 to \$30 per ton in the past three years. And for another, most of TVA's coal comes from strip mines, which are causing considerable environmental destruction and bitter resentment in some areas of Appalachia.

Although TVA officials take deserved pride in the fact that all their contracts with coal suppliers contain a clause requiring that strip mined lands be restored and revegetated, it's clear from testimony before a Senate committee last year that those requirements haven't always been met. "We can show anyone who would like to tour our area that TVA has never strictly enforced their regulations against certain operators", says J. W. Bradley, President of a group called Save Our Cumberland Mountains. Bradley went on to describe how "with every turn of the dozer's track and every bite taken by a large shovel, dragline, or front end loader, and by every blast of dynamite, we are furnished with more and more polluted water, fractured rock, loss of land and vegetation." Clearly, whatever requirements are imposed, TVA has been responsible for supporting a vast amount of strip mining, a practice which some regard as being unacceptably destructive.

Nuclear future

Those are some of the factors which have led TVA to seek a nuclear future, but it has found that it has in some respects traded one type of problem for another. So far, TVA has completed two of three units at the Browns Ferry nuclear plant, and four more units—at the Sequoyah Nuclear Plant and the Watts Bar Nuclear plant—are expected before 1980. All have experienced cost overruns, and the fire last year at Browns Ferry was a considerable setback. Started by an electrician checking for air leaks with a candle,

the fire knocked out several safety systems, came close to causing a nuclear accident (how close is a matter of debate), and has so far shut down the plant for about 16 months. According to Seeber, the fire has cost TVA about \$100 million in repairs and lost time.

TVA is, however, fortunate in one respect. There is little grass roots opposition to nuclear power in the region, and there have been few delays caused by anti-nuclear groups forcing public hearings and challenging power plant plans in court. In part, the lack of opposition can be explained by the fact that the area has always been associated with nuclear power through the Oak Ridge National Laboratory, which provided the plutonium and enriched uranium for the Manhattan Project and which is still a major nuclear facility.

Whatever the problems, TVA has so far shown no sign of backing off from its commitment to nuclear power. In fact, it has taken two steps to enhance its nuclear future. It is now investing heavily in uranium exploration in the Western states, with a view to securing uranium supplies. And it is a major partner in the liquid metal fast breeder reactor programme, providing some money and other support for the demonstration plant to be built at Oak Ridge.

TVA domination

Aside from the controversies over TVA's energy planning, there's one broad factor which seems to bother some people living in the Tennessee Valley. TVA's operations are so extensive that it literally dominates much of the economic life of the region, and there seems to have developed a feeling that it has become a faceless bureaucracy unresponsive to local needs. The chief problem is that TVA



Watts Bar nuclear plant

is a federal agency under the control, not of local legislative bodies, but of the US Congress 500 miles away.

Until very recently, TVA has been held in such affection by most residents of the Tennessee Valley—particularly those with long memories—that the remoteness of the control over the agency has been no problem. But

recent sharp increases in TVA's electricity rates in response to escalating coal prices have caused some alienation and frustration because there's no local body to which complaints can be addressed. The frustration was voiced on several occasions during last year's Senate hearings on TVA, the most provocative comment being the reference

to colonialism, but few suggestions for improving the situation were voiced.

Nevertheless, to most of the people in the region, TVA remains an instrument for economic progress. Certainly, few would deny that it has more than lived up to Roosevelt's hopes that it would reclaim the Tennessee Valley's land and people. □

US SPACE

Biological business boom

Sandy Grimwade reports from Washington on the progress of the Viking 1 experiments on Mars

AFTER more than five weeks of practically faultless operation the Viking 1 Mars lander continues to send a wealth of geological, meteorological, biological, chemical and visual information back to Earth. The only instrument which has failed to function correctly is a set of miniature seismometers which remains locked in the safe landing position, despite efforts to free it. The other teams of scientists have, however, been kept busy by the sheer volume of information being supplied to them.

Some of the most puzzling and to the general public most fascinating data have come from the three biology experiments and the related molecular analysis of the Martian soil. Although control and duplicate experiments—the *sine qua non* of all biological investigation—have not yet been done, speculation is rife as to the meaning of the results so far. The scientists involved are understandably cautious about delivering a yes-no answer to the question of life on Mars, when only a small portion of their programme is complete. The results so far are certainly not what would be expected if Earth-type organisms were present, nor are they explicable in terms of "simple" non-biological chemistry.

In the labelled release (LR) experiment, a mixture of ^{14}C -labelled nutrients in a small amount of water was added to a soil sample and incubated at about 15°C , which is $35\text{--}135^\circ\text{C}$ warmer than the Martian surface temperature. The totally unexpected result showed a massive and rapid release of radioactivity into the gas phase—presumably as carbon monoxide or dioxide. The radioactive counts released reached 4,500 in 10 hours, levelling off after 48 hours at about 8,500. A second dose of radioactive nutrients caused an initial burst followed by a drop in the released radioactivity, after which the graph

almost flattened out for the remaining four days of the experiment.

That "almost" is the tantalising part, because careful statistical analysis revealed a possible trend of very slow accelerating release of radioactivity. Unfortunately, the exigencies of operating one's laboratory by remote control across 200 million miles required the experiment to be stopped for performance of a control test with heat-sterilised soil. A later repetition of the experiment is planned with an incubation time of weeks or months rather than days. The results certainly do not look like anything one would expect from an Earth sample in the same situation. A chemical explanation involving oxidation of the labelled nutrients is at present the most popular explanation for the initial burst of activity.

The possibility that the Martian soil contains active oxidising agents was strengthened by the result from the gas exchange (GE) experiment. In this experiment the soil sample is either humidified or wetted with a concentrated "chicken soup" of amino acids, salts, vitamins and other nutrients, and the atmosphere is periodically monitored for changes in the concentration of gases by gas chromatography. The initial humidifying of the soil sample produced a remarkable release of oxygen which levelled off after several hours and remained stable for days. Wetting of the soil caused a rapid drop in the

atmospheric CO_2 , probably due to the formation of bicarbonate in the alkaline solution, and a slower drop in the oxygen, which is speculated to be due to oxidation of ascorbic acid which is present in the nutrients. At present, CO_2 is slowly being released into the atmosphere and the experiment is intended to continue for some time to see if any further changes occur. As the instrument is capable of detecting very small changes in hydrogen, methane, nitrogen and other gases, a wide range of possible biological activities could be detected.

The results of the GE and LR experiments are open to several more or less imaginative interpretations, and attempts are at present being made to duplicate the results in several Earth laboratories using simple catalysts and oxidising agents. The third experiment, the pyrolytic release experiment (PR), although perhaps the most complex technologically, is also the least equivocal when it comes to interpretation of the results. It is designed to detect the fixation of labelled carbon monoxide and dioxide in organic compounds. A sample of soil is incubated in a Martian atmosphere with added water vapour and irradiated with a simulated sunlight xenon lamp. After five days the sample is heated to 625°C to pyrolyse organic compounds which are trapped as vapours, and to drive off unreacted CO and CO_2 . This unreacted gas is passed through a radioactivity detector and forms the first "non-biological" peak. The organic vapour trap is then heated to 700°C to release and oxidise



Martian dune field

Photo: NASA

the remaining organic compounds which form the second, "biological" peak.

With a sterile sample a peak ratio of about 500 to 1 would be expected. The result from the Martian soil was about 75 to 1 due to the high "biological" peak. This is several times more active than non-sterilised soils from the dry valleys of Antarctica. The result from the control experiment with heat-sterilised soil is crucial in this instance, a high peak ratio indicating the possibility that biological

rather than chemical processes capable of fixing atmospheric carbon monoxide or dioxide in the presence of light are taking place in the soil. If life exists on Mars, this is what might be expected in the soil surface which is strongly illuminated and bathed in an atmosphere of about 95% carbon dioxide. The biologists would be even more encouraged if organic compounds were detected in the Martian soil.

Unfortunately, the initial run of the organic chemistry analysis was less

than satisfactory due to the temperamental soil sampling boom which apparently failed to deliver a full load of soil to the gas chromatograph-mass spectrometer. No organic compounds were detected. A full soil load has now been obtained and a further analysis is being carried out. If organic compounds are not detected this would be a blow, though not a fatal one, to the concept of life on Mars, and would render interpretation of the three biology experiments more difficult. □

USSR SPACE

Forward with the programme

Vera Rich reports on the latest Soviet space missions

"Soviet science considers the creation of orbital space stations with interchangeable crews as the main highway of mankind into space. They can form 'cosmodromes in space', launch pads for flights to other planets. Large-scale scientific laboratories will arise for the investigation of space technology and biology, medicine and geophysics, astronomy and astrophysics."

So said Mr Brezhnev in a recent statement. The idea was by no means new; it is a fundamental tenet of all Tsiolkovskii's work that such a station would and must precede manned exploration of the moon and planets. There are, indeed, some indications that Soviet space planners have at times been prepared to consider the alternative of manned exploration direct from earth—the argument that a lunokhod-carrying automatic spacecraft could carry out its survey work at "half the cost" of a manned mission, for example, suggests that the latter possibility had at least reached the costing stage. Now, the flight of the unmanned Luna 24 has repeated the task of Luna 16 by obtaining and returning with a sample of moon-rock, though from a depth of two metres and from *Mare crisium*, from which no samples had yet been obtained. Presumably present space plans still do not envisage cosmonauts venturing beyond earth orbit.

Whatever the overall timetable of the Soviet Union's space plan, though, many of the experiments now being conducted by the 7-week-old Salyut 5 mission suggest that the concept of a permanently manned space station is still well to the fore. These latter experiments fall into three main groups: geophysical and astrophysical observations, technological experiments and biological experiments. Of the technological experiments, some are directly concerned with processes and techni-

ques which would be required in the construction of a space station—notably the study of the behaviour of gases in liquids in space and, of some importance, a soldering experiment. Unlike the welding experiments of an earlier mission, the soldering, which uses a highly exothermal chemical reaction to produce the necessary heat, is aimed less at testing the cosmonaut's dexterity than at investigating the process itself. The welded metal specimens are to be returned to earth for further investigation of their structure.

The emphasis has clearly shifted to the properties of materials treated under conditions of weightlessness. Other experiments include the growing of crystals (also to be returned to earth), and the cooling of molten metals—bismuth, lead, tin and cadmium—to determine surface tension. These experiments will, of course, provide data of considerable theoretical significance: crystals grown under weightlessness, for example, should be free of convection-induced defects, and should, it is hoped, provide valuable comparative data for crystallographers and geologists. It is significant, however, that all these experiments are described in the official announcements as "technological", suggesting that their practical importance should not be minimised.

The medical problems of prolonged spaceflight are also receiving considerable attention. Apart from such obvious parameters as muscle tone, cardiovascular and respiratory performance, and loss of body weight—measured on the special "massometer"—the medical programme includes special monitoring of the cosmonauts in a "vertical" position (however that is to be interpreted), tests on reaction time in performing complex operations and manual control (real or simulated) of the spacecraft (the simulated control presumably relates to possible emergencies), and even possible variations in taste.

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Photo: Novosti

Before blast-off for Salyut 5

Press comment on the medical programme suggests a growing concern over the psychological effects of prolonged spaceflight. These would arise from a lack of external stimuli, the restriction of living space and general monotony—an aspect of spaceflight which will assume ever greater importance in future missions when the length of flight and the crew size increases. Aboard Salyut 5 are such simple prophylactics as recorded music (from folk to classical) and a varying make-up of the ration-packs.

Commenting on the radiation safety system used aboard the Salyut craft, Ludvig Palmbakh of the USSR Institute of General Genetics stated that the protective measures used are "well adapted" for work near Earth, since, if solar radiation rises to danger level, the crew can be quickly evacuated and brought back. For deep-space missions, however, "more active" means of protection would probably be required, possibly involving the deflection of high-energy particles by electromagnetic fields. The design of such systems entails considerable technical difficulty, so the fullest possible information is required both on the nature of the radiation and the actual hazard involved. The cytological and genetic experiments, using drosophila and hawksbeard, carried by Salyut 5, should provide further data on these hazards. □

IN BRIEF

Seabed hearings

The dispute between Greece and Turkey over the Aegean seabed is due to receive a hearing at the International Court of Justice at The Hague this week. Greece is seeking to prevent the Turkish oceanographic research ship *Sismik 1* from continuing its oil exploration, and wants a ruling on the limits of the continental shelf. In New York at the latest round of the Law of the Sea Conference, meanwhile, Dr Kissinger is reportedly threatening unilateral action by the US over exploitation of deep-sea minerals, on the regulation of which the conference has been deadlocked.

Pesticide dumping

In what Federal prosecutors in the US have described as the largest environ-

mental case ever brought, the Allied Chemical Corporation last week pleaded *nolo contendere* on 940 counts connected with the dumping of the pesticide Kepone in the James River. Fish in the river and in Lower Chesapeake Bay (where an Allied Chemical barge containing 250,000 tons of sulphuric acid capsized last week) were found to contain Kepone. The corporation could now face fines of about \$13 million.

Coal gasification 'option'

On the basis of a report it published last week, the UK National Coal Board (NCB) has decided not to resume, at least for the moment, applied research into underground coal gasification—the direct extraction of gases from coal seams burnt underground. The NCB identifies underground gasification as

a realistic energy option, however, and will continue monitoring international research efforts until costs—now put at 7–24 pence a therm—can be reduced to something nearer those associated with conventional mining techniques.

Cooperation in an international pilot scheme costing £10–15 millions could, the NCB feels, become worthwhile within five years or so. Outstanding problems of underground gasification include the poor quality of the gases produced, the lack of control over supplies, and the danger of seepage to the surface or into mineshafts and groundwater. During the 1950s Britain was among the pioneers of underground gasification research, which is now led by Belgium, Canada, the USA and West Germany. The Soviet Union operates a small-scale commercial facility.

YEARS ago, as a young father, I was often asked by my infant children for a bedtime story. On one such occasion, I gave a partially true account of a camping trip to a lonely spot where I was suddenly confronted, at dusk, by a ferocious animal, yellow-eyed and snarling. It was a wildcat! At this point in the narrative, the children screamed and hid beneath the covers. Soon they were sleeping soundly. Next evening, I asked what they wanted to hear in the way of a soporific monologue. The answer, to my surprise, was: *The Wildcat Story*. It was a best-seller for weeks. People love tales that make their flesh creep.

As time went by, I recognised many versions of the wildcat story in scientific writings. Whether or not these are true, their common property is that they are entrancing because they are "scary". One of the most fascinating is the recombinant DNA story. My grandchildren are too ecologically sophisticated to be terrified by wildcats. Perhaps I can startle them with an imaginary account of meeting up with a genetically-engineered *E. coli* cell.

According to a recent news report, many inhabitants of Cambridge, Massachusetts, are greatly worried by the possibility that "hot" *E. coli* cultures might escape into the sewers, nowadays called "the environment". Carelessness in the laboratories of Harvard University and the Massachusetts Institute of Technology was named as a cause that might lead to this disaster, which could be accentuated by the decrepitude of the facilities at Harvard. It was said that George Wald has explained the

danger to the Mayor of Cambridge, Massachusetts, Mr Vellucci, who has been an advocate of converting Harvard Yard into a parking lot. The

Wildcat story



THOMAS H. JUKES

mayor was apparently a ready listener, this being a rare case of a progressive public official giving ear to erudite eloquence. Perhaps, with the Mayor's attention diverted by colon bacilli, Harvard Yard may be saved.

The incident is somehow typical of a shift in attitudes towards scientific discoveries. Once we were eager to try new things, and none would say us nay. Today, dangers are recognised even before they have been shown to exist. If penicillin had been discovered in the 1970s instead of the 1930s, it

would probably have been kept out of the hospitals after preliminary tests had shown it to be potentially allergenic, and the testers had been sued for medical malpractice.

Somehow I have a feeling that anything *E. coli* could do to us has already been done during the millions of years that this organism has lived in countless numbers within the intestinal tracts of vertebrates. It has a set of genes that code for transfer RNA molecules. But these molecules have different base sequences from those in human tRNAs, although both sets of tRNAs function similarly. The differences indicate that the sets have evolved separately for hundreds of millions of years. Why has there been no mixing? Are all the transformations phenotypic? Would the new, human-tailored bugs take over the sewers? Or would they stand about as much chance as a hothouse orchid in a patch of good healthy ragweeds?

If Mr Vellucci prevails, we are not likely to find out. I would be a bit peeved if I were a young bacteriologist, being held back by the old guard who have made it to the top. However, many young scientists seem quite satisfied to join, or even to excel, the elderly prophets of doom.

Now that Viking has landed, we can brace ourselves for an airing of the greatest of all wildcat stories. It seems that a capsule of unsterilized Martian soil came back to Earth, swarming with weird organisms that gobbled up atmospheric nitrogen. The first news we heard was that millions of people were dying east of the Urals. . . .

news and views

Concentration factors and radiological protection

from Pamela M. Bryant

THE behaviour of plutonium in the environment is of topical interest especially in view of proposals in several countries of the world to embark on power programmes involving the use of fast breeder reactors. Knowledge is currently expanding as a result of studies on plutonium present in the environment from nuclear weapons tests and disposal of wastes from nuclear fuel reprocessing plants. From a knowledge of the levels of plutonium activity in relevant environmental materials it is possible to estimate the associated radiation exposure of humans. When that knowledge is sufficiently broad to quantify the behaviour of plutonium in inter-related environmental materials, it is possible to predict the approximate level of radiation exposure of humans corresponding to the postulated additions of plutonium to the environment.

In a paper in this issue of *Nature* (page 745) reporting the results of measurements of plutonium concentrations in the water of Kwajalein lagoon in the Marshall Islands, Noshkin *et al.* examine published data on plutonium concentrations in water and fish from nearby and distant locations. This examination leads the authors to conclude that all the available data on plutonium in fish are seemingly inconsistent and that, if the available Enewetak and Kwajalein data are correct, the availability to all fish and invertebrates of plutonium, from whatever source, depends more upon the type of environment than on plutonium levels in that environment. They state that such a conclusion would greatly affect future plans for discharging low activity transuranic wastes to the marine environment. They also say that it would force them to concede that the concept of a plutonium concentration factor for fish (the ratio of the concentration of plutonium in fish to its concentration in the surrounding water) is meaningless.

In a preliminary report on levels of plutonium isotopes and ^{241}Am in various tissues in plaice from the north-eastern Irish Sea, Pentreath and Lovett (this issue of *Nature*, page 814)

include data on discharges of these nuclides from the nuclear fuel reprocessing plant at Windscale, Cumbria. Plaice is a fish of commercial importance in the area; it is monitored regularly, as part of the established control procedure of the Ministry of Agriculture, Fisheries and Food (MAFF), primarily to ensure the radiological safety of the discharges in public health terms. Pentreath and Lovett comment that in view of the fluctuating discharges a steady-state burden will not exist for fish caught close to the pipeline; thus, an accurate estimate of the concentration factor between fish and seawater cannot be made. The concentration factor would, in any case, depend on the location of the seawater sample and the movement of the local population of plaice.

Yet another complication in the estimation of concentration factors is evident from recent reports which suggest a preferential biological availability of ^{238}Pu over $^{239,240}\text{Pu}$ in terrestrial and aquatic organisms. The reports were noted with interest by Beasley and Fowler (page 813 of this issue of *Nature*) in a paper describing plutonium isotope ratios in polychaete worms. These authors found that these deposit-feeding marine worms, living in sediments contaminated with plutonium isotopes from a variety of sources, did not preferentially accumulate ^{238}Pu over $^{239,240}\text{Pu}$. They conclude that until the reasons for the disparities become clear general statements regarding preferential biological availability of ^{238}Pu over $^{239,240}\text{Pu}$ should be viewed cautiously.

The limitations of values for concentration factors between water or sediments and marine organisms are well recognised by persons using them for prediction purposes in the field or radiological protection and carrying out research programmes connected with control procedures for waste discharges.

A basic policy applied to the control of radioactive discharges to the marine environment from nuclear establishments in the United Kingdom is that the government departments respon-

sible authorise discharges at levels below those which correspond by calculation to the dose limits for the general public recommended by the International Commission on Radiological Protection (ICRP). The authorised limits are set on the basis of the need to do what is reasonably practicable, having regard to cost, convenience and the national importance of the subject, to reduce doses far below the ICRP dose limits. The environmental consequences of such discharges of radioactivity are also borne in mind, although it is generally found that the public health considerations are more limiting than considerations relating to environmental resources.

The first stage in determining discharge levels corresponding to ICRP dose limits, for example when a new discharge is being considered, involves modelling of the environmental pathways likely to be of greatest importance. Such modelling seeks to estimate the long-term average level of radiation exposure, under steady-state conditions, corresponding to the postulated disposal practice. Where an element of doubt exists, pessimistic values of concentration factors are used. In the early stage of an operation, discharge of no more than a small fraction of the level indicated by this modelling is permitted; at this stage research additional to control monitoring is undertaken to check the predicted relationships.

In the particular case of plutonium discharges from Windscale, monitoring is carried out by the MAFF Fisheries Radiobiological Laboratory of materials which include the important elements of human food chains such as fish, shellfish and the edible seaweed *Porphyra umbilicalis*, as well as seawater and sediment whose role as a "sink" for plutonium introduced into the sea is well recognised. The object of this study is to answer some of the questions posed by the introduction of a long-lived radionuclide into the marine environment. The expected results of this study include improved understanding of the concentration factors between environmental materials in the region and their limita-

tions. Control of public health, however, is exercised by the direct measurement of radioactivity in those environmental materials responsible for human exposure; in the case of actinides discharged from Windscale the most important measurements concern plutonium and americium in the edible fraction of Irish Sea plaice taken

from the sea area most liable to contamination. It is difficult to understand, therefore, why the uncertainty as to the validity of the concept of concentration factors for fish expressed by Noshkin *et al.* should lead to their conclusion that future plans for discharging low activity transuranic wastes to the marine environment would be affected.

Enkephalin: the latest instalment

from Leslie Iversen and Raymond Dingleline

The 5th International Narcotic Research Conference was held in Aberdeen on July 19–22, 1976. The full proceedings of the meeting will be published by North-Holland in September under the title *Opiates and Endogenous Opioid Peptides*.

THE central effects of morphine and opiate-like drugs have been known for thousands of years, but only in the past five years has real progress been made in understanding the actions of these drugs on the brain, or the significance of the existence of specific opiate receptors in normal brain function. The first catalytic breakthrough was the discovery by Pert and Snyder¹ of a simple, sensitive assay for brain opiate receptors. Equally exciting was the report by Hughes *et al.* last December² of the identification of two naturally occurring pentapeptides in brain—the enkephalins—whose properties mimic those of morphine, and this was the focus of attention at the recent meeting in Aberdeen.

Although the identification of the enkephalins has since been confirmed³, it has also become apparent that there are several other, larger, naturally-occurring peptides that share similar pharmacological properties. Most of these peptides, now known generically as the “endorphins”, derive from the 91-amino acid pituitary peptide β -lipotropin. Thus several groups have now reported that a pituitary peptide corresponding to the C-terminal portion of β -lipotropin (residues 61–91, known as C-fragment of lipotropin or β -endorphin) has morphine-like properties in various test systems^{4–6}. Similar properties are exhibited by other naturally occurring fragments of β -lipotropin, for example the C¹-fragment (residues 61–87, also known as γ -endorphin) and α -endorphin (residues 61–76)⁷. Met-enkephalin (Tyr-Gly-Gly-Phe-Met) consists of residues 61–65 of β -lipotropin, and all

of the above peptides share this common sequence at their N-terminal region. However, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) does not seem to be derived from β -lipotropin, and there may be yet more morphine-like peptides still to be characterised. A. Goldstein (Stanford University) reported at the meeting that it seems unlikely that the β -lipotropin derived peptides can account for all of the opioid material extractable from pituitaries, and A. Wahlström (University of Uppsala) reported on an opioid peptide from human cerebrospinal fluid that appears to be chemically different from all known fragments of β -lipotropin. In addition, C. Pert (National Institute of Mental Health) reported that normal human blood contains a low molecular weight opioid, named “anodynin”, which is not an enkephalin⁸. The anodynin content of rat blood was abolished after hypophysectomy, strongly suggesting pituitary origin.

All this is, to say the least, a little confusing. Are the pentapeptides in brain really the naturally occurring morphine-like substances? Should one perhaps regard the pituitary peptides and brain enkephalins as belonging to completely different functional systems, or could the enkephalins merely represent breakdown products of the larger peptides? If so, it would be a unique situation among peptide hormones for their precursor molecules to exhibit strong physiological activity, since the larger endorphins seem to be more potent than the enkephalins in most test systems. The enkephalins are, however, rapidly destroyed by peptidases in brain and other tissues⁹, and the metabolic stability of the larger endorphins may account for their dramatic and prolonged pharmacological actions. In support of this A. Pert (National Institute of Mental Health) reported that the simple chemical derivative D-Ala²-Met-enkephalinamide, which has half the affinity for the opiate receptor of Met-

enkephalin but is resistant to enzymatic degradation, produced powerful long-lasting analgesia in rats when injected into the brain. Other synthetic analogues of the enkephalins that are metabolically stable were reported by D. Smyth (National Institute for Medical Research, Mill Hill), and they seem likely to become useful research tools.

A disappointing finding for interested clinicians was that prolonged treatment with the endorphins seems to induce tolerance and dependence, just like other opiates. The clearest evidence for this came from E. Wei (University of California), who reported that following the chronic infusion of very small amounts of β -endorphin or Met-enkephalin into the brain (using an ingenious implanted osmotic mini-pump), rats exhibited typical withdrawal signs when challenged with naloxone (see also ref. 10).

Further progress in identifying central sites for the analgesic effect of opiates was provided by H. Takagi (Kyoto University), who reported that microinjection of morphine into the nucleus reticularis gigantocellularis resulted in naloxone-sensitive analgesia and mimicked the inhibitory effect of systemic morphine administration on spinal lamina V cells. H. Akil (Stanford University) demonstrated that endorphin levels in rat brain rise during a stress-induced analgesic state, and postulated that environmental situations that activate the endogenous pain-inhibitory system¹¹ may involve the opioid peptides. Of potential importance also is the recent finding by Simantov and Snyder¹² that enkephalin levels are increased in the brains of morphine-tolerant rats.

Many speakers described the close similarity between the actions of morphine and the endorphins in brain and peripheral targets. Thus the endorphins produce a naloxone-sensitive analgesia when injected into the brain (see also refs 13 and 14), have characteristic morphine-like actions on the guinea pig myenteric plexus and mouse vas deferens preparations, and display other behavioural opiate-like effects such as catatonia in rats and locomotor activity in mice. In addition, all the endorphins displayed a respectable affinity for binding to opiate receptors, but here some curious anomalies were described. N. Birdsall (National Institute for Medical Research) showed that the displacement by morphine or Met-enkephalin of a radiolabelled opiate agonist followed different kinetics from their displacement of labelled antagonists (see also refs 15 and 16). Met-enkephalin was less effective in competing for binding sites for labelled naloxone than in competing for its

own binding sites in brain, and morphine was much less potent in competing for labelled enkephalin binding sites than in displacing ^3H -opiates. These differences might be explained by multiple receptor conformations¹⁷, but H. Kosterlitz (University of Aberdeen) proposed the existence of a family of opiate receptors, on the basis of the binding anomalies and variations in rank order of potency for a series of opiates when tested in different peripheral assays.

In terms of cellular responses it was clear that the enkephalins closely mimic morphine. Thus, B. Hamprecht (Max-Planck-Institut für Biochemie, Martinsried) found that very low concentrations of enkephalin stimulate cyclic GMP formation in cultures of neuroblastoma $\times$ glioma cells, and W. Klee (National Institute of Mental Health) that enkephalins inhibit basal and prostaglandin-stimulated cyclic AMP formation in the same cells (see also refs 18 and 19). K. Minneman (University of Cambridge) reported a similar stimulation of cyclic GMP formation by morphine and the enkephalins in slices of rat striatum, although high concentrations of the peptides were needed because of their metabolic instability²⁰. Several groups have studied the responses of single neurones in different brain regions to iontophoretically applied enkephalin, and in general these peptides mimic the inhibitory actions of morphine²¹⁻²⁴, although specific excitatory actions have been observed on Renshaw cells²⁵. Intracellular records from spinal interneurons and motoneurons by W. Zieglansberger (Max-Planck-Institut für Psychiatrie, Munich) suggested that the inhibitory effect of morphine and enkephalin may be due to blockade of ionic conductance at chemically excitable Na^+ channels, an hypothesis similar to that of Snyder's group¹⁷. Intracellular recordings from morphine-sensitive neurones in the myenteric plexus by R. North (Loyola University) however, showed that at this peripheral site opiates may hyperpolarise cells by causing an increase in membrane conductance.

Several other new pieces of information relevant to understanding the possible physiological function of the endorphins were presented. R. Simantov (Johns Hopkins University) and J. Hughes (University of Aberdeen) found that the regional distribution of enkephalin in brain is similar to that of the opiate receptor itself: with high levels, for example, in corpus striatum and hypothalamus and negligible amounts in the cerebellum. Hughes also described a calcium-sensitive release of enkephalin-like material from brain synaptosome preparations in response to depolarising concentrations

of potassium. Progress in discovering possible "endorphinergic" synapses will be aided by two other technical developments: the autoradiographic mapping of the anatomical distribution of opiate receptors (reported on by M. Kuhar, Johns Hopkins University), and the mapping of possible endorphin-containing neuronal pathways in the brain by immunohistochemistry. The latter method has recently been successfully applied by T. Hokfelt and reported at the Collegium Internationale Psychopharmacologium meeting in Quebec last month. Further instalments of this bulletin may be expected before long!

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Farmington: an Apollo meteorite

from David W. Hughes

FARMINGTON Township, Washington County, Kansas, is famous, at least to meteoritists, as the place where an L5 chondrite fell to ground in June, 1890. The meteorite itself is famous because it has a uniquely short cosmic-ray exposure (or "radiation") age, having left its parent body a mere 25,000 years ago. This latter fact is most significant because it means that the orbit the meteorite had as it intersected the Earth was its original orbit, the one it obtained immediately after release from its parent body. This orbit therefore gives a direct clue to its place of origin.

Meteorite ages are obtained by look-

ing at the accumulated spallation products and nuclear tracks produced in the body of the meteorite due to the penetration of galactic cosmic rays. This irradiation usually begins as soon as the meteorite is ejected from its parent body, so the radiation age equals the transit time between that body and Earth. On average this is 10^7 years. Now every time a meteorite passes close to a planet its orbit changes, and a pass as far away as 10 planetary radii will alter the direction of motion of the meteorite by a few degrees. Opik (*Adv. Astron. Astrophys.*, **4**, 301; 1966) calculated that this happens about every 5×10^5 yr so Farmington, which had had a separate existence in the Solar System for a twentieth of this time, was most probably on its pristine, unaltered orbit. This would differ slightly from the orbit of the parent body because of the random velocity given to Farmington on ejection but as this velocity is generally less than a few hundred metres per second the change is only marginal. So with Farmington (and Farmington alone, as other meteorites have been in the Solar System much longer and have greatly altered orbits) there is a real chance of finding out exactly where it came from. To do this its orbit has to be calculated and Boris Levin and Alla Simonenko (Academy of Sciences of the USSR, Moscow) and Edward Anders (University of Chicago) have done just this in a recent paper in *Icarus* (**28**, 307; 1976).

They enlisted the help of the Kansas and Nebraska Historical Societies who diligently went through 280 different newspapers from 91 counties published around June, 1890, looking for reports of the fall. Thousands of people over a 300×300 km area saw the bolide and 144 news items appeared in the press. There were many vivid descriptions: "a bright meteor passed swiftly to the north-west and burst into glittering fragments high in the air"; "it was a ball of fire as large as a table. It had a trail like a comet and it wobbled like a kite"; "its path could be discerned by the smoke that followed it". To some it sounded like the firing of a cannon, to others it had a "long rumbling rolling noise like thunder". Close to the impact point the ground shook just as in an earthquake. The most startled person was a Mr J. H. January who was "breaking the prairie sod" when the 85 kg meteorite fell almost vertically some 150 m in front of him and made a hole with nearly vertical sides.

None of these descriptions of the Farmington bolide contains any quantitative data on the azimuths and angular heights of the beginning and end points of the fireball. But near the point of fall anyone seeing the meteorite would be looking along the trajectory and the

fireball would appear to be stationary and in the direction of the apparent radiant. Just such an effect was observed from Washington, Kansas, 7.1 km south of Farmington where the meteorite was also seen to be just below the Sun. The explosion and extinction happened after the passage over Washington, at an end height of about 10 km. The zig-zag course reported by some people near Washington was due to a physiological effect. The eye can only follow a moving object if its angular velocity is less than 30° s^{-1} . At greater velocities the eye pursues the object in jumps (the so-called saccadic movement). As this jumping was seen, the object must have been moving at about 5 km s^{-1} or somewhat slower.

Combining all the observations, Levin, Simonenko and Anders conclude that the apparent radiant was 20° west of south at an azimuth of 60° , giving Farmington a very steep trajectory through the atmosphere. The beginning and end heights were very similar to the Pribram and Lost City meteorites (98, 87 km and 13, 17 km respectively), the only other two meteorites to have known orbits (these being obtained from photographs taken from networks of cameras). The pre-atmospheric velocity must have been in the range 11.3 to 22 km s^{-1} . The lower bound is the Earth's escape velocity, the upper one being due to the fact that meteors moving faster than 22 km s^{-1} suffer complete ablative destruction. Using velocities in this range and the apparent radiant position obtained from the eye-witness reports the authors calculate a set of pre-collision orbits for the meteorite. They find that the meteorite struck the Earth at the ascending node of its orbit ($\Omega=275^\circ$) and that the orbit had a small semi-major axis (between 1 and 1.3 a.u.) and a low inclination (5 to 10°). The eccentricity was around 0.21 and the argument of perihelion between 240 and 270° . The aphelion was at 3.0 a.u., in the densest part of the asteroid belt, and the perihelion could not have been less than 0.4 a.u. (interestingly chemical analysis showed that ^3He and ^4He had not been lost by the meteorite during its lifetime supporting the argument against a small perihelion). Fortunately all these results are insensitive to errors in the apparent radiant.

The facts point towards Farmington being a fragment from the collision between an Apollo asteroid and some other meteorite or asteroid. The Apollos are a short lifetime (10^7 – 10^8 yr) group of asteroids which have Earth-crossing orbits. They are thought by some to be too rare to provide all the stony meteorites. If they do, then the undiscovered Apollos should exceed the known Apollos by one or two orders

of magnitude.

Of the 11 known Earth-crossing asteroids with encounter velocities below 22 km s^{-1} , Hermes, 1862 Apollo and 1865 Cerberus have orbits which fall within the error limits of the Farmington orbital elements, however none can be singled out definitely as the parent body. The uranium–helium age of Farmington is 5.5×10^8 yr (Heymann, *Icarus*, 6, 189; 1967) indicating that it was reheated extensively, as were all L chondrites, in the collision that broke up the parent body. This collision must have involved an

unusually large projectile which deposited more energy per unit mass than a typical cratering event. The remnants of the body could comprise a highly fragmented Hirayama or Flora family with a steep mass distribution.

So amidst a plethora of theories concerning asteroidal origin Farmington's place of birth seems to be known and was most probably an Apollo asteroid, this fact coming to us by courtesy of the careful scrutinising of the 1890 files of such worthy papers as the *Atchison Patriot*, the *Omaha Daily Bee* and the *Wymore Weekly Wymorian*.

EMBO ribosome workshop

from Richard Brimacombe

The fifth EMBO workshop on ribosomes took place in Brussels from August 2–6, 1976 under the organisation of Dr Alex Bollen of the Université Libre de Bruxelles.

THE ever-increasing complexity of ribosomology was reflected in the large number of participants (about 150), but the meeting was also characterised by a notable change of emphasis in the range of topics discussed, which was largely a result of the increasing contribution of chemical and physicochemical studies to the subject, at least in the case of prokaryotes. Progress over the past two years was summarised in the opening lecture by the organiser of the previous EMBO meeting (C. G. Kurland, Uppsala University), who laid particular stress on the developments in physical chemistry, which has become not only concerned with the structural aspects of the ribosome problem, but is also beginning to be concerned with the question of the specificity of the translation process in terms of kinetics.

One of the physicochemical methods now being used successfully to investigate ribosome structure is neutron scattering: results obtained with this technique were presented by P. Moore (Yale University), who, by making reconstituted ribosomes containing two deuterated proteins, has been able to estimate the distances between centres of mass of a number of pairs of proteins within the *E. coli* 30S subunit. The technique also indicates that many proteins are far from spherical in shape. Neutron scattering gives information about the overall shape and size of the subunits, and has been used in addition to probe the distribution of RNA and protein within the subunits; it seems that the central cores of the subunits are rich in RNA, particularly in the case of the 50S, whereas the

surface regions are richer in protein (M. Koch, University of Louvain).

The neutron data are not yet sufficiently advanced to be compared in detail with the data from another very topical technique—the direct electron-microscopic demonstration of protein-specific antibodies bound to the ribosome surface. This method has produced two models of the *E. coli* ribosomal subunits, one of which was presented by G. Stöffler (Berlin) and the other by J. Lake (University of California, Los Angeles). Both protagonists arrived armed with an impressive array of hideously-coloured plastic ribosomal sculpture, and there was some lively discussion of the differences between the two models, which rather tended to obscure the fact that despite these differences there is substantial agreement, with regard both to the overall shapes of the subunits and to the details of the protein arrangement. In particular, there can now be no doubt that many proteins have very irregular and elongated shapes within the ribosome.

Physical chemistry is now being applied to the kinetics of the translation process. Ten years ago the question of codon–anticodon recognition did not seem to be a problem, but now it is by no means simple when considered in detail (J. Ninio, Paris; H. Grosjean, Brussels). The crux of the matter seems to be the degree of “stickiness” of the interaction between a codon and a potential anticodon; the correct anticodon will have a longer sticking time, and thus more chance of leading to amino acid incorporation, than an incorrect anticodon. The ribosome therefore needs to have time to make up its mind what to incorporate, an idea supported by some results from A. Spirin (Poustchino), who showed that stimulation of the rate of the translocation process by addition of

Mercury's magnetic field

THE origin of Mercury's magnetic field has recently become a subject of considerable research interest. Still debatable is whether the dipolar field (less than a thousandth of that on Earth) comes from a currently active dynamo or is a relic of earlier fields (or, of course, is some combination of these). We wish to take up some of Peter Smith's points in an earlier *News and Views* article (*Nature*, **260**, 578; 1976) on this subject.

If the field observed comes from remanent magnetisation only, the first question that has to be decided is down to what depth this magnetisation exists. Mercury's core, if not generating a magnetic field, would probably still be above the Curie point (Solomon, *Icarus* (in the press)), so it is reasonable to think only of a shell of material with modest magnetic susceptibility. Goldstein (*Nature*, **258**, 175; 1975) showed that a dipole moment results, in general, in such a shell after it has been magnetised in the field of an internal, now extinct, dipole. Stephenson, in a quantitative analysis of the problem (*Earth planet. Sci. Lett.*, **28**, 454; 1976) obtained an ancient surface polar field of less than 10 Oe only if the volume percentage of iron in rocks of a 243 km-thick mantle were 5%—more than a hundred times the typical value in lunar basalts. No support for such an anomalously high iron content comes from the Mariner 10 photometric observations (Hapke, *et al.*, *J. geophys. Res.*, **80**, 2431; 1975). Srnka has recently carried out a similar, though more detailed calculation (*E&S*, **57**, 271; 1975), from which he concludes that an ancient surface field of about 16 Oe was required along with a volume percentage of iron of about 3.0%. We currently believe that on the

basis of this work, the remanent magnetisation hypothesis for the field is not a likely one.

We admit that these calculations could be significantly modified; Solomon, for instance has pointed out that if the segregation of metals from silicates is incomplete in the mantle and crust of Mercury, the resulting increased thermal conductivity could lead to a thicker shell below the Curie temperature, somewhat reducing the required magnitude of the ancient magnetising field. Furthermore, if the iron content were as high as Stephenson assumes, then ferromagnetic effects might dominate.

There are, on the other hand, additional difficulties for remanent magnetisation models; among them is the observation of Murray *et al.* (*J. geophys. Res.*, **80**, 2508; 1975) and others, that craters such as the Caloris basin must have modified the planetary structure to depths greater than 100–200 km, approximately the thickness of the shell in which remanent magnetisation would be acquired. Cratering could thus severely disrupt any extant magnetisation pattern (Wasilewski, *Moon*, **9**, 335; 1974).

The other major alternative is a currently active dynamo for which a necessary but not sufficient requirement is a molten core—what evidence do we have for this? Solomon and others have pointed out that cosmochemical models not including a substantial heat source predict core solidification within about 1 billion years after infall. That would result in a contraction of Mercury's radius of some 18 km (Solomon, *op cit.*), which is not observed—indirect evidence that a molten core may exist. Proposals for heat sources include the retention of some

U and Th in the core after differentiation; the presence of about 156 p.p.b. ⁴⁰K; presence of elements such as S in the core, which would lower the solidus temperature; and models which distribute the gravitational heating over more of the planet's history.

Finally, there is the remote possibility that the field of Mercury is due to remanent magnetism from an external source field. Sharpe and Strangway (*Geophys. Res. Lett.*, **3**, 285; 1976) propose a cool model in which a 100 km shell of a few per cent iron cools rapidly below 800 °C in a field of some 10 Oe. In their calculation, they suggest that if Mercury accreted cold (at about 300 K; as opposed to 1,200 K which is usually used as the base temperature) the present-day field could result from fossil remanent magnetisation retained in the inner half of the planet.

Peale, in this week's *Nature* (page 765), proposes an experiment which would contribute to a solution of the origin question. The dynamical behaviour of Mercury will be slightly affected by the existence of a liquid core; measurements of the amplitude of libration in longitude, the obliquity to the ecliptic and two functions of moments of inertia should suffice. None of these measurements would be easy—the last two would require tracking of artificial satellites, the first two would require the establishment of some form of astronomical observatory on Mercury.

The origin of Mercury's magnetic field will probably not be resolved without additional visits to the planet.

M. L. GOLDSTEIN
N. F. NESS
P. J. WASILEWSKI

GTP caused a concomitant increase in misreading rate.

Another novel feature of this meeting was the devotion of a whole day to the question of ribosomal genetics. Large numbers of new mutant ribosomal proteins were described, which should prove useful in both genetic and functional studies, and some very impressive work on the ordering of individual genes within the *E. coli* chromosome was reported by S. Jaskunas and L. Lindahl (University of Wisconsin). Transducing phages carrying segments of bacterial DNA were isolated and the genes these contained could be identified using a DNA-dependent cell-free protein-synthesising system. The arrangement of the genes within the DNA segments was analysed with the help of restriction enzymes,

and genes for rRNA, several factors, and many ribosomal proteins have at least been partially ordered in this manner.

Activity in these new areas meant that less time than usual was available for discussion of the more traditional aspects of ribosomal research. Little was heard in Brussels on the primary structures of ribosomal proteins and RNA, in order to avoid repetition of the extensive reports in this field which had been presented at the International Congress in Hamburg during the previous week. A number of delegates described protein-protein cross-linking, RNA-protein cross-linking and affinity labelling results, and here the picture of the *E. coli* ribosome is becoming more and more complex. This is an obvious consequence of the irregularity

of the protein shapes; as long as it was possible to think of the ribosome as a neat array of ping-pong balls, it was sufficient to identify which proteins were involved in a cross-link or affinity label experiment. Now that the ribosome has become a plate of spaghetti, researchers will be forced to push the analysis one stage further and identify the amino acid residues within the proteins which are involved in the reaction in order to be able to interpret the results fully, and this is not an easy task. For the same reason, the functional investigation of the *E. coli* ribosome has become more complex; more and more proteins are becoming implicated in each individual ribosomal function, and active participation of the rRNA is also now being frequently invoked, although here there is a notable

lack of hard evidence. Nevertheless, various promising experimental approaches were reported, which should in due course converge on a clearer understanding of ribosomal function.

Finally, the eukaryotes had a short session to end the meeting. This session did not really do justice to the large amount of work now being done on the eukaryotic ribosome, which, roughly speaking, has reached the stage at which the *E. coli* work stood four years ago, in that (for example) ribosomal proteins are being isolated and characterised. Perhaps for this reason, the eukaryotic research seemed deceptively simple and straightforward at the moment, although the present state of the *E. coli* ribosome can leave nobody with any illusions as to what lies ahead in the eukaryotic field. □

Familial dysautonomia

from Felicia Axelrod, Thomas W. Mittag and J. P. Green

The Familial Dysautonomia Foundation sponsored a conference on familial dysautonomia and related diseases at New York University Medical Center on May 14, 1976.

FAMILIAL dysautonomia is a rare autosomal recessive disorder characterised by specific dysfunctions in the autonomic nervous system and the sensory nervous system. Prominent manifestations include postural hypotension; excessive sweating; diminished perception of pain, temperature, hypoxia, and taste—the last associated with an absence of fungiform papillae in the tongue. The corneal reflex is absent and the deep tendon reflex diminished. After intradermal injection of histamine, the axon flare, one of Lewis's triple responses, does not occur. The patients can cry but there are no overflow tears because the lacrimal glands function poorly. Methacholine restores the tendon reflex and the histamine response and stimulates the lacrimal gland, but conscientious symptomatic and supportive therapy is the only means to prolong life.

An unusual characteristic of the disease is that some organs show supersensitivity to sympathomimetic and parasympathetic agents, as though denervated. A diminished number of unmyelinated nerve fibres as reported by A. A. Aguayo (McGill University) can account for some of these signs and symptoms. Similarly, J. Pearson (New York University) reported that dorsal root ganglia and sympathetic ganglia show a reduced number of normal neurones and the sympathetic

ganglia contain a large number of cells resembling undeveloped neuroblasts. Additional evidence of peripheral nerve defects was presented by N. Grover-Johnson (New York University) who showed that blood vessels have a diminished number of autonomic nerve ending in these patients. By contrast the parasympathetic ganglia (ciliary and submaxillary) appear normal under the microscope (Pearson) even though it has long been known that in dysautonomic patients parasympathetically innervated structures such as the iris and lacrimal gland are supersensitive to parasympathomimetic drugs. E. Krikun and A. A. Smith (New York Medical College) presented evidence that an isozyme of choline acetyltransferase was deficient in the basal ganglia of a patient dying of the disease, suggesting a basis for the parasympathetic defect. The apparent developmental arrest of unmyelinated axons and certain sympathetic neurones in familial dysautonomia led to investigations into a possible defect in nerve growth factor or other trophic factors. F. B. Axelrod (New York University) reported that nerve growth factor activity in the serum of these patients was within the normal range when measured by bioassay (the ability to stimulate growth of chick ganglion cell dendrites). Yet a recent report (Rogers *et al.*, *Pediatric Res.*, **9**, 384; 1975 (Abstr. 764)) showed greater than normal concentration of the β -subunit of nerve growth factor in dysautonomic serum, measured by radioimmunoassay, but normal activity by bioassay. It was suggested (T. W. Mittag, Mount Sinai School of Medicine) that one possible explanation of this paradox is that patients have, in addition to the normal nerve growth factor, an abnormal nerve growth factor inactive in the chick bioassay but detectable by radioimmunoassay, which interferes with the action of normal nerve growth factor on human cells. There was general agreement that continuing studies of trophic factors that regulate the growth and development of autonomic and sensory nerves could be fruitful, perhaps leading to treatment and to prenatal diagnosis.

Although the disease is almost exclusively restricted to Ashkenazi Jews (in whom its frequency is 1 in 10,000) it was reported that some of the same signs and symptoms are present in other diseases such as idiopathic orthostatic hypotension (I. B. Black, Cornell University Medical College and A. Rubenstein, Mount Sinai School of Medicine) and diabetes mellitus (N. Grover-Johnson). The New York University group also reported that 75% of dysautonomics showed pathological changes in the renal glomeruli.

An understanding of the cause of

familial dysautonomia will provide increased understanding of the development of the autonomic and sensory nervous systems. And, in turn, progress towards understanding the molecular basis of familial dysautonomia could come from investigations of other diseases manifesting autonomic dysfunction such as idiopathic orthostatic hypotension and diabetes mellitus.



A hundred years ago

IN connection with the general introduction of the now celebrated Liberian coffee plants into most of the coffee-producing countries, as noticed by Dr. Hooker in his recently issued report on Kew Gardens, we may draw attention to what our consul says on the decrease of the production of coffee in Cayenne. The kind there cultivated is the Mocha, which at one time was an important staple of the colony, the country being especially adapted for its cultivation. This valuable product of Cayenne, although temporarily abandoned, is not lost to the world; the trees continue to thrive in a wild state, and may be reclaimed hereafter. There are thousands of coffee trees interspersed in the forests of the inhabitable part of the colony which have been abandoned for years. They attain a height of about fifteen or sixteen feet, with a circumference, a few feet from the ground, of thirty inches; they are rich in foliage, but do not bloom. The coffee tree also appears to be safe from the ravages of insects, whereas many other trees suffer vitally from this evil.

THE proposal to submerge a portion of North Africa by means of a canal from the Gulf of Gabes, letting the water of the Mediterranean westwards over the lake region of Djerid, seems from the facts detailed by MM. Roudaire and Dupuis to be not only a practicable, but also likely to turn out a remunerative undertaking. Owing to the comparatively small area it is proposed to submerge, the meteorological changes which the submersion would occasion can only be slight, strictly local, and altogether beneficial in their general tendency—differing absolutely in all these respects from the meteorological changes which would result from the submersion of the western portion of the Sahara, proposed some time ago. From this latter project it would follow, owing to the great extent of the water surface which would thus overspread the Western Sahara, and its proximity to the Atlantic, that the present disposition of the lines of atmospheric pressure would be seriously altered, a result necessarily attended with changes in the prevailing winds and currents of the North Atlantic, seriously affecting international interests in a manner which our present knowledge does not enable us in any way accurately to predict.

from *Nature*, **14**, August 24, 361; 1976.

articles

The distribution of stars around a massive black hole

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The stellar density distribution around a massive black hole at the centre of a spherical star cluster is determined from a simple scaling argument. The distribution is obtained by examining the net diffusion timescales associated with star and energy transport in a spherical, N -body system in dynamic equilibrium. The same analysis is also used to determine the stellar density profile in the halo of a star cluster. The approximate distribution obtained here for a relaxed, N -body system with (or without) a central, massive black hole agrees qualitatively with the results of detailed numerical calculations.

THE suggestion^{1,2} that accretion on to a massive black hole ($M \sim 10^3 M_\odot$) might be responsible for the X-ray emission observed from globular clusters has stimulated new interest in the dynamical consequences of a collapsed object at the centre of an N -body system. Some confusion has arisen, however, concerning the resulting steady-state, stellar distribution near the black hole. In his pioneering analysis^{3,4}, Peebles used a simple scaling argument to suggest that the stellar density varied as

$$n(r) \sim n_a(r/r_a)^{-9/4}$$

in the region $r_t < r < r_a$. Here n_a is the central density of the isothermal cluster core,

$$r_a \sim GM / \langle v^2 \rangle$$

is the 'capture' radius within which core stars with velocity dispersion $\langle v^2 \rangle$ are bound to the black hole of mass M , and $r_t \ll r_a$ is the radius at which stars tidally disrupted by the black hole. All stars are assumed to have the same mass, m , with $m \ll M \ll M_c$, where M_c is the total cluster mass. Recently, more detailed calculations (J. Bahcall and R. Wolf, unpublished; A. P. L. and S. L. S., unpublished) have indicated that the appropriate density law near the black hole is more aptly described by the relation

$$n(r) \sim n_a(r/r_a)^{-7/4}$$

where the magnitude of the exponent decreases slowly with decreasing r because of the consumption of low angular momentum stars (A. P. L. and S. L. S., unpublished; J. Frank and M. J. Rees, unpublished).

Here we use a simple scaling argument to obtain the approximate density law $n(r) \propto r^{-7/4}$ and provide a simple explanation for the discrepancy between our derivation and the original argument suggested by Peebles. In particular, we emphasise the distinction and general inequality between the net energy diffusion timescale and the net particle diffusion timescale in an N -body system in dynamic equilibrium. We use the same

analysis to determine the density distribution in the cluster halo surrounding the isothermal core. (The halo solution applies whether or not the cluster contains a black hole.) Thus, by careful delineation and the appropriate use of the two different timescales mentioned above, we obtain, in a few lines, the approximate stellar density distribution throughout a relaxed, spherical, N -body system containing a central black hole. As becomes evident, the analysis applies equally to any sufficiently massive compact object (for example, a super-massive star) at the centre of a star cluster.

Diffusion timescales in N -body systems in dynamic equilibrium

As a conservative, N -body system of identical point stars (mass m) approaches equilibrium stochastically through mutual gravitational encounters, stellar velocities change, and thus both energy and angular momentum are exchanged, on a relaxation timescale

$$t_R \sim \langle v^2 \rangle^{3/2} / (G^2 m^2 n)$$

If the entire stellar system were confined in a spherical container with perfectly reflecting walls, the system would achieve thermal equilibrium with no net flow of stars or energy out of the system. (Here we assume that the container radius is sufficiently small that the ratio of the central density to the density at the wall < 709 . In this case the equilibrium is stable, and runaway evolution through the 'gravothermal instability' will not occur (see refs 5, 6).) If, however, the system is not confined by rigid walls and if, in addition, there exists a star 'sink' at its centre (for example, a black hole), significant departures from thermal equilibrium will exist near the centre and in the outer halo of the stellar system. The steady-state behaviour of the cluster in these distinct, non-thermal regions will be one of dynamic equilibrium, characterised by a constant net flux of stars and energy out of the two regions. As energy is continually exchanged between stars, a given group of stars occupying the radial zone between r and $2r$ in a non-thermal region will be scattered out of the zone in a time t_R . The timescale associated with the net diffusion of stars (or energy) across a sphere at r may, however, be considerably longer, since stars (and energy) from neighbouring radial zones are scattered into the zone between r and $2r$ in roughly the same timescale, t_R . Clearly, no physical quantity can be transported from one zone to the next on a net rate exceeding $\sim t_R^{-1}$. Only by examining the boundary layers of a given non-thermal region can one determine the net flux of stars and energy into the region. In general, the net energy and net particle diffusion timescales will not be equal and only the minimum of the two will equal t_R in any region in dynamic equilibrium.

Let the net star and net energy diffusion timescales at radius r in the cluster be denoted by $t_s(r)$ and $t_u(r)$, respectively. For a steady state, both the net star flux S and net energy flux U are

constant and independent of radius. If $n(r)$ is the stellar density and $E(r)$ is the mean energy (per unit mass) at r , then the number of stars between r and $2r$ is $\sim nr^3$ and in the steady state we may write

$$S \sim \frac{n(r)r^3}{t_s(r)} = \text{constant} \quad (1a)$$

$$U \sim \frac{n(r)r^3 E(r)}{t_U(r)} = \text{constant} \quad (1b)$$

In thermal equilibrium the constants on the right-hand sides of equation (1) are zero. In dynamic equilibrium, they are generally non-zero and equation (1) gives

$$t_U(r) \propto t_s(r)E(r) \quad (2)$$

demonstrating the simple but important result that stars and energy do not, in general, diffuse on the same timescale in systems in dynamic equilibrium. The proportionality constant in equation (2) is obtained by analysis of the boundary conditions of the region of interest, as will be illustrated below. The resulting steady-state star distribution will always be valid whenever the local value of τ_R ($\sim 10^8$ – 10^9 yr in the cores of typical globular clusters) is much less than the timescale for the dynamic evolution of the cluster ($\sim 100 \tau_R$ for evaporation (see below) and $\sim 10^{11}$ yr for a $10^3 M_\odot$ black hole to swallow its own mass of stars in a globular cluster (A.P.L. and S.L.S., unpublished; J. Frank and M. J. Rees, unpublished)).

Density distribution in a spherical cluster containing a central black hole

Equations (1a, b) and (2) may be used to determine the density distribution in a spherical cluster containing a massive black hole. The cluster may be conveniently divided into four distinct regions.

Region I: central cusp; $r_t < r < r_a$

This innermost region consists of negative energy stars bound to the central black hole. In steady state, the consumption of stars at r_t is balanced by a net inward flux of stars originating from the isothermal core at $r \gtrsim r_a$. Since bound stars of negative energy are removed at r_t , there exists a net outward flux of energy from this region, thereby allowing distant stars to move closer to the black hole to replenish the depleted star distribution. In this region $E(r) \sim -GM/r$, so from equation (2)

$$t_U \propto t_s r^{-1}$$

The absence of outgoing stars at r_t implies that the net diffusion time for stars to move from $2r_t$ to r_t equals the timescale for their (negative) energy to decrease by a factor of two. Thus $t_U(r_t) \sim t_s(r_t)$, and from equation (2)

$$t_U(r) \sim (r_t/r)t_s(r) \quad (3)$$

For $r > r_t$, t_U is clearly the minimum timescale and can be equated to the relaxation timescale, $t_U \sim t_R \sim \langle v^2 \rangle^{3/2} / (G^2 m^2 n)$. For bound stars near the black hole $\langle v^2 \rangle \sim GM/r$, so equation (1b) yields

$$n(r) \propto r^{-7/4} \quad (4)$$

The above result has been obtained previously (J. Bahcall and R. Wolf, unpublished; A. P. L. and S. L. S., unpublished) by detailed analysis of the steady-state diffusion of stars toward a central black hole. Peebles obtained equation (1a) and assumed the relation $t_s \sim t_R$, thereby overlooking the distinction between t_U and t_s and the fact that nearly equal ingoing and outgoing star fluxes at r result in a reduced net inward flux.

His resulting solution

$$n(r) \propto r^{-9/4}$$

thus requires $t_U(r) < t_R(r)$, which forces stars at small radii $r' \ll r$ (and so small $t_R' \ll t_R$) to move towards r and results in a net outward star flux in violation of the boundary conditions. In region III of the globular cluster, t_s is the minimum diffusion timescale, rather than t_U , and there t_s can be equated to t_R legitimately.

Region II: isothermal core; $r_a < r < r_c$

In this region, which contains most of the cluster mass, thermal equilibrium prevails with a Maxwellian velocity distribution law and an isothermal density distribution law. To lowest order $S \sim U \sim 0$. In each relaxation time $t_R(r_a)$, only those few high velocity stars on the tail of the distribution with velocities exceeding the escape velocity

$$\langle v_{esc}^2 \rangle^{1/2} = 2\langle v^2 \rangle^{1/2}$$

(roughly 1% of the total number) evaporate^{7,8} from the core and populate the halo. The outer radius of the core r_c is determined by equating the local relaxation time $t_R(r_c)$ to the mean evaporation timescale, $\sim 100 t_R(r_a)$; stars with $r > r_c$ cannot thermalise sufficiently rapidly to maintain thermal equilibrium in the presence of escaping stars. Since

$$t_R(r) \propto n^{-1}$$

in the isothermal core, we have

$$n(r_c)/n_a \sim 10^{-2}$$

or

$$r_c \sim 10 / (12\pi G n_a m / \langle v^2 \rangle)^{1/2}$$

where we use the standard isothermal profile in the region $r_a < r < r_c$. Detailed numerical calculations⁹ confirm this qualitative picture.

Region III: halo; $r_c < r < r_{e2}$

The cluster halo is populated by high energy, bound stars ejected from the isothermal core. Halo stars move in highly eccentric orbits and, in each orbital period, penetrate the dense core where they suffer repeated, small angle gravitational encounters. Since all halo stars move with nearly the same velocity during their periastron passage through the core, they all experience roughly the same r.m.s. energy boost, ϵ_2 , each period, independent of their energy E . Consequently, ϵ_2 is given by the (random walk) relation

$$\epsilon_2 \sim \left(\frac{t_d}{t_R} \right)^{1/2} E = \text{constant} \quad (5)$$

where

$$t_d \sim 2\pi G M_c / (2|E|)^{3/2}$$

is the ($\sim$ Keplerian) orbital period of a halo star of energy $E \sim -GM_c/r$. Equation (5) implies that

$$t_R(r) \propto r^{-1/2}$$

In steady state, a constant net flux of stars moves outwards in the halo, providing a constant escape rate of positive energy stars ejected from the outermost halo or 'fringe' beyond $r \gtrsim r_{e2}$. At $r_{e2} \sim GM/\epsilon_2$, stars can acquire positive energy in just one additional passage through the core. Since at r_{e2} there are no ingoing stars, we have, in analogy to the discussion for

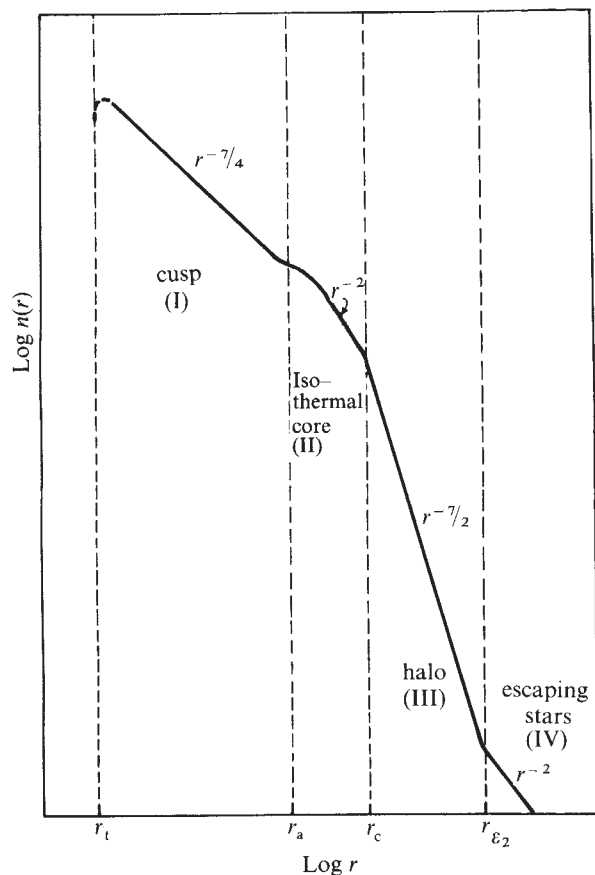


Fig. 1 The stellar density $n(r)$ as a function of radius r in a spherical cluster containing a massive, central black hole (see text for discussion of the different regions).

region I, $t_U(r_{\epsilon_2}) \sim t_S(r_{\epsilon_2})$. Equation (2) then implies

$$t_U(r) \sim (r_{\epsilon_2}/r) t_S(r) \quad (6)$$

In the halo, $r_c < r < r_{\epsilon_2}$, t_S is now the minimum timescale; and so $t_S(r) \sim t_R(r)$. This disparity in diffusion timescales merely reflects the fact that energy is nearly conserved in a cluster experiencing stellar evaporation. Substituting

$$t_S(r) \sim t_R \sim r^{-1/2}$$

into equation (1a) yields the halo density distribution law

$$n(r) \propto r^{-7/2} \quad (7)$$

Region IV: escaping stars; $r_{\epsilon_2} < r < \infty$

In this non-thermal region, unbound, non-interacting stars with energy $E \sim \epsilon_2$ move radially outwards to infinity with constant velocity

$$v_\infty \sim (\epsilon_2)^{1/2}$$

A steady state requires $S \sim n v_\infty r^2 = \text{constant}$, hence

$$n(r) \propto r^{-2} \quad (8)$$

Similarly, $U \sim S \epsilon_2 \sim \text{constant}$. The above results derived for regions II–IV pertain with or without a central black hole, provided $M \ll M_c$. Detailed numerical calculations of the outer regions of a spherical cluster confirm¹⁰ equations (7) and (8).

The entire run of the stellar density in a spherical cluster containing a central black hole is illustrated schematically in Fig. 1. For a $10^3 M_\odot$ black hole at the centre of the globular cluster NGC7078 (M15), where¹¹ $\langle v_0^2 \rangle^{1/2} \sim 10 \text{ km s}^{-1}$, $n(r_a) \sim 5 \times 10^4 \text{ pc}^{-3}$ and $m \sim M_\odot$, the critical radii separating the different regions are (approximately) $r_t \sim 3 \times 10^{-7} \text{ pc}$, $r_a \sim 3 \times 10^{-3} \text{ pc}$, $r_c \sim 3 \text{ pc}$, and $r_{\epsilon_2} \sim 10^3 \text{ pc}$. Since r_{ϵ_2} is larger than the tidal radius of the cluster in the galactic field, the stellar distribution in the outer halo will be modified as the energy required for escape falls below zero. By observing the stellar density distribution in the core of globular clusters with a large telescope, black holes with masses $> 10^3 M_\odot$ may be detected (J. Bahcall and R. Wolf, unpublished).

We thank E. E. Salpeter for several useful discussions, and the NSF for support.

Received March 25; accepted July 7, 1976.

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Plutonium levels in Kwajalein Lagoon

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Reported plutonium levels in fish from both Kwajalein and Enewetak lagoons, suggest that Kwajalein Lagoon contains significantly more plutonium in its environment than would be expected from worldwide fallout levels alone, although quantities of plutonium greater than fallout concentrations have not been detected in the lagoon water. If there is no reason to reject the published fish data, then individuals on Kwajalein Atoll who supplement their diet with foods from the local marine environment may have plutonium body burdens similar to the low levels predicted for individuals on similar diets at Enewetak Atoll.

KWAJALEIN ATOLL (9°N 167°40'E) is located in the western (Ralik) chain of the Marshall Islands. In the same island chain and some 300 miles to the north-west is Enewetak Atoll (11°20'N 162°20'E), one of the United States nuclear testing sites during the 1940s and 1950s. It has been reported¹ that no immediate debris from the nuclear tests at Enewetak and Bikini (another Pacific test site) was deposited at Kwajalein. Measurements during 1972 and 1973 between 5 and 15°N, over a wide range of longitudes, indicated concentrations of between 0.22 and 0.44 pCi m⁻³ for ^{239,240}Pu in Pacific surface water^{2,3}. The mean ^{239,240}Pu concentration in the surface waters of Kwajalein and other Pacific atoll lagoons located in this latitude band, therefore, would be expected to be similar to the surface oceanic level (0.34 ± 0.11 pCi m⁻³).

Table 1 Lognormal median concentrations of $^{239,240}\text{Pu}$ in fish tissues (pCi g^{-1} dry weight) collected at Enewetak and Kwajalein atolls*

	Enewetak Atoll (all samples)		Kwajalein Island		Kwajalein Atoll Meck Island		Enewetak Island	
All fish species								
Bone	0.038	(24)	0.086	(3)				
Muscle	0.013	(123)	0.023	(11)	0.01	(6)	0.53	(3)
Gut	0.45	(6)	0.051	(3)			0.42	(2)
Surgeonfish								
Muscle	0.028	(28)	0.02	(3)			0.96	(1)
Gut	0.019	(26)	0.03	(2)			0.43	(1)
Mullet								
Muscle	0.014	(25)						
Gut	0.75	(19)						
Goatfish								
Muscle	0.008	(21)						
Gut	0.093	(18)						
All other fish								
Bone	0.038	(24)	0.086	(3)				
Muscle	0.009	(49)	0.024	(8)	0.01	(6)	0.40	(2)
Gut	0.25	(44)	0.14	(1)			0.41	(1)

*See refs 1 and 5.

†Values in parentheses are numbers of samples analysed. Average muscle and gut values in pCi g^{-1} dry weight can be converted to average pCi g^{-1} wet weight by dividing by 3.5.

This is not true of Enewetak Lagoon. In late 1972, the average measured concentration of $^{239,240}\text{Pu}$ in the lagoon surface water³ was 39 pCi m^{-3} , approximately 100 times the level predicted from worldwide fallout. Clearly, some components of the atoll contaminated by fallout debris during the tests are contributing substantial amounts of $^{239,240}\text{Pu}$ to the lagoon water masses. The difference in $^{239,240}\text{Pu}$ concentrations between the two lagoons is a direct reflection of the activity levels in the environments of the two atolls. It also follows that if plutonium uptake in living organisms is expressed in terms of a concentration factor (the concentration of plutonium in the tissue of the marine organism divided by its concentration on an equivalent weight basis in the surrounding water), invertebrates, fish, or other marine organisms can be useful as indicator species of the level of environmental contamination.

Schell and Watters⁴ have given $^{239,240}\text{Pu}$ concentrations in various organs of selected marine organisms collected at Enewetak and Kwajalein atolls. They concluded, however, that the plutonium and americium concentrations in the convict surgeon fish from Enewetak Atoll, for example, are not significantly higher than those measured at the control station, Kwajalein Atoll. The mantle and muscle tissues of a clam (*Tridacna* sp.) collected in the south-eastern region of Enewetak Atoll were, moreover, found to contain only one-sixth as much plutonium as those of a *Tridacna* sp. collected at Kwajalein Atoll⁴. On the other hand, the viscera and kidney of the same Enewetak Atoll clam had higher concentrations than those of the Kwajalein Atoll specimen.

In an assessment of the possible plutonium dose to man from food and other environmental pathways, Wilson *et al.*⁵ compiled, in the form of lognormal median concentrations, all the plutonium survey data collected at Enewetak Atoll during 1972 and 1973 (ref. 1). The concentration values for all the fish muscle samples taken are reproduced here in Table 1, together with similar lognormal mean concentrations compiled from the same survey¹ data for fish bone and gut from Enewetak Atoll and muscle, bone, and gut concentrations, also from the same survey data¹, for fish collected off three islands of Kwajalein Atoll (W. R. Robison, private communication). Wilson *et al.*⁵ found no significant differences in mean plutonium concentrations among four fish groups, which included, among others, planktonic and detritus feeders, grazing herbivores, bottom-feeding carnivores, and pelagic carnivores. Accordingly, they based their predictions of exposures to

plutonium from ingestion of marine foods on the mean concentration of plutonium in the muscles of all the fish taken from the lagoon of Enewetak Atoll. Significantly, however, the $^{239,240}\text{Pu}$ in the lagoon water ranged from 1 to 96 pCi m^{-3} at the fish sampling sites¹.

Radionuclide concentration independent of environment?

The data of Schell and Watters⁴, together with those of Table 1, show that average levels of $^{239,240}\text{Pu}$ in fish bone, muscle, and gut from Enewetak Atoll are similar to, or even lower than, those of the fish indigenous to the control station, Kwajalein Lagoon. There are several possible important conclusions that can be derived from these data. The first is that fish collected for consumption by man will contain, on average, essentially the same concentrations of plutonium radionuclides regardless of the source or level of plutonium in the local environment. Obviously, such a conclusion would greatly affect future plans for releasing low-level transuranics to the marine environment. Furthermore, it would force us to concede that the concept of a plutonium concentration factor for fish is meaningless. Also, it conflicts with a large body of plutonium concentration data for Atlantic fish species that derive their plutonium body burdens from worldwide fallout levels in the Atlantic Ocean. For example, for a number of Atlantic species, including bottom feeders, water-column feeders, and large predators⁶⁻⁸, a lognormal median of all available bone concentration data is only $1 \times 10^{-4} \text{ pCi g}^{-1}$ (wet or dry), 900 times less than that for the bones of the Kwajalein fish (Table 1). A similar large discrepancy remains when the concentrations in the muscle of the Atlantic and Kwajalein fish are all normalised to an equivalent weight basis (wet or dry).

Plutonium levels in the Atlantic waters (where some of the fish were caught) range only between 0.2 and 1.1 pCi m^{-3} , according to Bowen *et al.*⁸. Calculating a concentration factor from the data for Atlantic fish and water and using a value of 0.4 pCi m^{-3} as the assumed mean plutonium level from fallout in the Kwajalein Lagoon, provides values of between 0.2 and $1.0 \times 10^{-4} \text{ pCi g}^{-1}$ for the bone of fish for this lagoon. These values are orders of magnitude lower than the lognormal median concentrations in Table 1. This discrepancy cannot be accounted for by any possible differences related to trophic levels or feeding habits. A similar calculation also yields large dis-

crepancies between predicted and measured concentrations for fish muscle.

Excess plutonium at Kwajalein

An alternative explanation to account for these discrepancies would be that Kwajalein Lagoon contains significantly more plutonium in its environment than would be expected from worldwide fallout levels alone. To test this possibility, 551 unfiltered water samples were collected during May and June of 1975 from the locations shown in Fig. 1. During June, two samples were also collected outside the Atoll in the north equatorial surface waters, to provide information on the plutonium levels in the open ocean in this region. Unfortunately, our schedule did not allow time to collect fish on Kwajalein Atoll.

The water samples were analysed for $^{239,240}\text{Pu}$ and ^{137}Cs using the methods described in refs 9 and 10. Our analytical results are shown in Table 2. Although the lagoon was not sampled in great detail, the data are sufficient to show that the average $^{239,240}\text{Pu}$ concentration in Kwajalein Lagoon (0.45 ± 0.21 pCi m^{-3}) is nearly the same as the mean for the surface water of the ocean in the area, and that it agrees reasonably well with the levels previously predicted from worldwide fallout.

At the time the Kwajalein water samples were being processed we were also participating in an intercomparison exercise with Woods Hole Oceanographic Institution to determine $^{239,240}\text{Pu}$ levels in replicate surface-water samples from one location in the North Atlantic. The mean value for eight samples from Woods Hole (V. T. Bowen, unpublished) was 0.63 ± 0.16 pCi m^{-3} , whereas ours for 10 samples was 0.70 ± 0.30 pCi m^{-3} . This analytical agreement (as well as that in other national and international intercomparisons that we have completed) lends a measure of confidence to our data. In addition, these comparative data show that, as expected from worldwide depositional data¹¹, average plutonium levels are somewhat lower in Kwajalein Lagoon than in North Atlantic surface waters. The average $^{239,240}\text{Pu}/^{137}\text{Cs}$ ratio of 0.0032 for Kwajalein Lagoon is also in good agreement to the average value for open ocean, in contrast to the ratio of 0.07–0.12 in the Bikini and Enewetak lagoons³. This illustrates that both the $^{239,240}\text{Pu}$ and the ^{137}Cs content of the lagoon are derived from worldwide fallout.

We are still without an obvious explanation for the discrepancy in the fish-tissue data; that is, why should the Kwajalein fish, in a region of the equatorial Pacific contaminated only by worldwide fallout, have significantly higher body burdens of plutonium than fish in the North Atlantic which is also contaminated only with worldwide fallout? Furthermore, why do the Kwajalein Atoll fish have the same or even higher body burdens than the fish at Enewetak Atoll, where higher levels of plutonium are found in the lagoon? And why were the concentrations in Enewetak Lagoon fish not correlated with those in the specific local environment where they were sampled, or with feeding habit or trophic level? A cursory search of the literature surprisingly revealed only one other relevant fallout report—the plutonium level in the body of a single marine fish

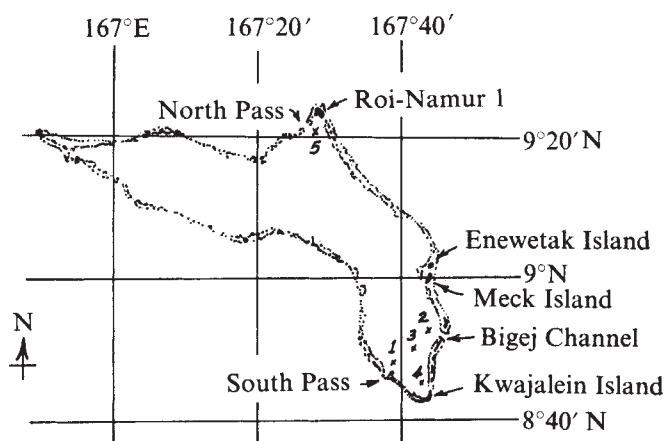


Fig. 1 Locations of sampling sites (see Table 2).

in fallout-contaminated waters of the Pacific¹². The plutonium concentration in this sample and the computed concentration factor are closer to those from the North Atlantic fish than to those from Kwajalein. On the other hand, plutonium levels in tissues from catfish from Trombay¹³, in plaice from the vicinity of Windscale¹⁴, and in several species from areas of Thule¹⁵ are higher than those in the tissues of the North Atlantic fish, and close in value to those from Kwajalein and Enewetak. But as the last three sets of data are from areas known to have local plutonium contamination from reprocessing plants (and one nuclear accident), they support our argument that levels of plutonium in fish reflect local environmental levels.

To sum up, it appears that available plutonium data from fish tissues are inconsistent. If we accept the available Enewetak and Kwajalein Atoll data as correct, then we can only conclude that the availability to all fish and invertebrates of plutonium from fallout, or for that matter from any local source, depends more on the type of environment than on the plutonium levels in that environment. The data could also suggest that coral atolls may have specific biogeochemical processes that regulate the availability of plutonium regardless of levels in the environment or in food. On the other hand, if we assume that concentrations in fish and invertebrates are proportional to those in the environment, then we can only conclude that many of the atoll fish data from the laboratories involved in analytical programmes^{1,4} are in error. Since there is no other evidence at this time to refute the analytical results from the atolls, we can only urge caution in applying concentration factors measured in one marine area to predict approximate plutonium levels in fish from other marine areas. In addition, it seems, on the basis of the published data^{1,4} and the summary assembled in Table 1, that individuals on Kwajalein Atoll who supplement their diet with foods from the local marine environment may have plutonium body burdens similar to the low levels predicted⁵ for individuals at Enewetak on similar diets.

Table 2 Concentrations of $^{239,240}\text{Pu}$ and ^{137}Cs (pCi m^{-3}) in seawater in Kwajalein Lagoon and two locations in north equatorial waters

Station*	Depth (m)	Collection date	$^{239,240}\text{Pu}$	^{137}Cs	$^{239,240}\text{Pu}/^{137}\text{Cs}$
1	Surface	5/10/75	0.33 (20)†	137 (3)	0.0024 (20)
1	44	5/10/75	0.87 (13)	144 (5)	0.0060 (14)
2	Surface	5/08/75	0.29 (27)	131 (5)	0.0022 (27)
3	Surface	5/08/75	0.26 (24)	127 (3)	0.0020 (24)
3	47	5/08/75	0.33 (25)	129 (3)	0.0026 (25)
4	Surface	5/08/75	0.54 (16)	129 (4)	0.0041 (16)
5	Surface	6/14/75	0.52 (18)	132 (4)	0.0039 (18)
10°26'N 166°31'E	Surface	6/15/75	0.36 (32)	132 (3)	0.0027 (32)
11°16'N 165°45'E	Surface	6/15/75	0.53 (23)	143 (4)	0.0037 (23)

*Kwajalein stations are shown in Fig. 1.

†Values in parentheses are the 1σ counting errors expressed as percentages of the listed values.

This work was performed under the auspices of the US Energy Research and Development Administration.

Received April 14; accepted June 26, 1976.

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Identification of phage SP01 proteins coded by regulatory genes 33 and 34

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Bacillus subtilis phage SP01 induces polypeptides of 24,000 and 13,500 daltons that are bound to a form of RNA polymerase that directs phage "late" transcription *in vitro*. These proteins are the products of SP01 genes 34 and 33, regulatory cistrons that control "late" gene expression *in vivo*.

GENE expression during the lytic cycle of *Bacillus subtilis* phage SP01 is controlled in a temporally defined sequence¹. Phage "early" genes are transcribed almost immediately after infection and this RNA synthesis is directed by the sigma subunit of the host RNA polymerase. Next, phage "middle" genes are turned on at 4-5 min into the lytic cycle; this transcription is activated by the protein product of SP01 regulatory gene 28 (refs 2 and 3). Gene 28 is now known to code for a phage-induced protein that binds to *B. subtilis* RNA polymerase⁴ and that apparently directs specific transcription *in vitro* in the absence of sigma factor⁵⁻⁸. A host protein, δ , has been described that improves the accuracy of middle-gene transcription by the phage-modified RNA polymerase.

Activation of SP01 "late" genes between 8 and 14 min into the lytic cycle seems to require a further modification of RNA polymerase. Pero *et al.*⁹ have shown that a form of the *B. subtilis* transcriptase containing phage-induced subunits of 24,000 (termed V) and 13,500 (termed VI) daltons preferentially copies 'late' genes *in vitro*. The work reported here establishes that these phage-induced subunits are coded by SP01 genes 34 and 33, regulatory cistrons that control late gene expression *in vivo*^{2,3}. An accompanying article⁹ demonstrates that the purified protein products of genes 33 and 34 and host delta factor act synergistically to direct the synthesis of late RNA by core RNA polymerase.

Induction of polypeptides V and VI by mutants

As an initial step in investigating the possible relationship of phage-induced polypeptides VI and V, to genes 33 and 34, the synthesis of these proteins in bacteria infected with wild-type SP01 was examined. Cells of a non-suppressing (*su*⁻) strain of *B. subtilis* were infected with wild-type phage and pulse labelled with ³⁵S-methionine at an intermediate time in the lytic cycle. RNA polymerase was

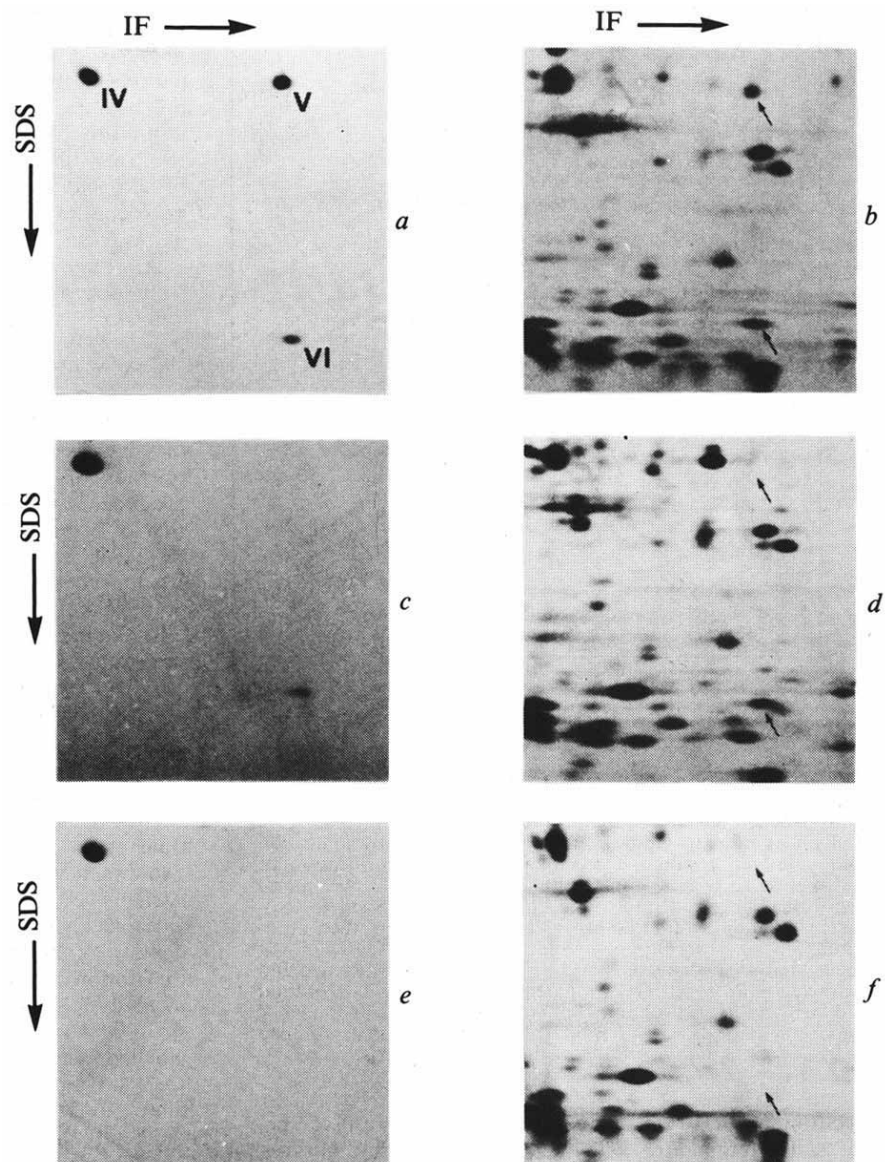
isolated from an extract of the infected cells by gel filtration and phosphocellulose chromatography. The enzyme was radiochemically highly purified at this stage of purification and contained essentially only host subunits of RNA polymerase and previously identified phage-induced proteins that copurify with polymerase¹⁰. Phosphocellulose-purified polymerase was then analysed by the two-dimensional gel electrophoresis procedure of O'Farrell¹¹; radioactive enzyme was denatured in urea and subjected to isoelectric focusing in the first dimension and sodium dodecyl sulphate (SDS) slab gel electrophoresis in the second dimension. Figure 1a is an autoradiogram of a region of the two-dimensional gel that contained a 26,000-dalton polypeptide termed IV (the product of gene 28)⁴, polypeptide V (24,000) and polypeptide VI (13,500). The approximate isoelectric points of proteins IV, V and VI were 5.4, 7.3 and 7.4 respectively. The identification of these proteins was confirmed by a mixing experiment in which the radioactive phosphocellulose-purified polymerase was coelectrophoresed in two dimensions with unlabelled phage-modified enzymes from a previous study⁵.

High resolution two-dimensional gel analysis also made possible the identification of polypeptides V and VI in crude extracts of SP01-infected bacteria. Radioactive protein extracted from bacteria infected with wild-type phage was subjected to two-dimensional electrophoresis. Figure 1b is an autoradiogram of a region of the gel corresponding to that of Fig. 1a and containing polypeptides IV, V, and VI. Since the relative position of spots varied somewhat from gel to gel, it was necessary to identify the phage-induced subunits of RNA polymerase by means of a mixing experiment (not shown) in which radioactive enzyme of Fig. 1a was coelectrophoresed with the crude extract of Fig. 1b (and with extracts of mutant-infected bacteria described below). Polypeptides V and VI were well resolved from other radioactive proteins and are designated with arrows. Polypeptide IV on the other hand, was partially occluded by another intensely labelled protein and is not designated in the figure.

To examine the effect of a mutation in gene 34 on the synthesis of the phage-induced subunits of polymerase, the *su*⁻ bacteria were infected with the nonsense mutant *susF4* (refs 2 and 3) and radioactively labelled at an intermediate time in the lytic cycle. Two-dimensional analysis revealed that polypeptide V was specifically lacking in both phosphocellulose-purified polymerase (Fig. 1c) and unfractionated extract (Fig. 1d) from the mutant-infected cells. This finding is consistent with the possibility that subunit

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Fig. 1 Two-dimensional electrophoresis of subunits IV, V and VI in RNA polymerase and in crude extracts from non-suppressing bacteria infected with wild-type and mutant phage. The *su⁻* strain was a methionine prototrophic derivative of *B. subtilis* Mu8u5u6 (*ade⁻*, *leu⁻*, *met⁻*)¹² and was obtained from A. L. Sonenshein. Cells were grown at 37 °C in a modification of 121A medium¹³ containing leucine (50 µg ml⁻¹) and adenine (100 µg ml⁻¹). Cultures of the bacteria (150 ml) were labelled by the addition of 2 mCi of ³⁵S-methionine (high specific activity; prepared as described before¹⁴) 10–14 min after infection with the mutant (obtained from S. Okubo) and wild-type phage indicated below (multiplicity of infection 5). RNA polymerase (*a*, *c* and *e*) was purified from 0.3 g of infected cells as described before⁴, concentrated by trichloroacetic acid precipitation¹⁰ and dissolved in "lysis buffer" (ref. 11, modified to contain 2% ampholines (LKB) pH range 3–10). Crude extracts (*b*, *d* and *f*) were prepared from 6 mg of infected cells as follows. Infected cells were suspended in 0.2 ml of buffer containing 0.01 M Tris-HCl (pH 7.9), 0.01 M EDTA and lysozyme (300 µg ml⁻¹), incubated for 1 h at 0 °C and then disrupted by brief sonication. Next, MgCl₂ (to 0.02 M), RNase and DNase (to 50 µg ml⁻¹) were added. After low speed centrifugation, the resulting supernatant fluids were incubated at 0 °C for 1 h and then added at room temperature to 0.43 ml of the modified "lysis buffer" containing an additional 235 mg of urea. Samples of RNA polymerase (~10⁵ c.p.m.) and of crude extract (~2.3 × 10⁶ c.p.m.) in the "lysis buffer" were then subjected to isoelectric focusing as described by O'Farrell¹¹ but with the following modifications. The gels contained 5.4% polyacrylamide⁴ and 1.9% ampholines (LKB) comprised of 1.5% pH range 3–10, 0.2% pH range 9–11, 0.1% pH range 5–7 and 0.1% pH range 4–6. The lower electrode (cathode) solution contained 0.4% ethylenediamine. The isoelectric focusing gels were then subjected to electrophoresis in a second dimension through discontinuous SDS slab gels as described⁴. (It should be noted that the separation of subunits IV and V in discontinuous SDS gels is strongly dependent on the commercial brand of SDS (J. Pero, J. Nelson and T.D.F., unpublished results and ref. 15) and that the separation achieved here using a crystalline form of SDS from BioRad is less than was previously obtained using SDS in powdered form from BioRad (refs 5 and 6) and less than can be obtained using SDS from Pierce Chemical Co. (unpublished results).) Each picture is an autoradiogram¹⁶ (100 h exposure) of a region of the two-dimensional gels to which subunits IV, V and VI migrate. The following samples



were subjected to electrophoresis: *a*, RNA polymerase from bacteria infected with wild-type SP01; *b*, crude extract of bacteria infected with wild-type SP01; *c*, RNA polymerase from bacteria infected with the mutant *susF4*; *d*, crude extract of bacteria infected with the mutant *susF4*; *e*, RNA polymerase from bacteria infected with the

mutant *susF14*; *f*, crude extract of bacteria infected with the mutant *susF14*. The IF (isoelectric focusing) arrows point in the direction of increasing pH; the SDS arrows point in the direction of decreasing molecular weight. The arrows in the autoradiograms of crude extracts indicate the positions of subunits V and VI.

V is the product of gene 34.

Figure 1*e* and *f* shows the effect of a nonsense mutation in gene 33, *susF14* (refs 2 and 3), on the induction of the phage-induced subunits. Surprisingly, *su⁻* bacteria infected with the gene 33⁻ mutant failed to synthesise detectable quantities of both polypeptides VI and V (although in experiments comparable with Fig. 1*f* faint traces of a protein in the position of V were observed). This finding is consistent with an assignment of subunit VI as the product of gene 33 if, as discussed below, the *susF14* mutation in gene 33 somehow interferes with the synthesis of polypeptide V, the putative product of gene 34.

Although mutations in either gene 33 or gene 34 alone interfere with the global transcription of late genes^{2,3}, close inspection of Fig. 1 reveals certain protein species whose synthesis is blocked uniquely in either the gene 33⁻ or the gene 34⁻ mutants. While this has yet to be investigated, the kinds of arguments used to explain the pleiotropic effect

of *susF14* on the synthesis of polypeptide V (see below) could be applied here.

The ability to detect polypeptides V and VI in crude extracts by means of two-dimensional gel analysis made it possible to determine whether the synthesis of these proteins is induced at a middle time in the phage lytic cycle, as would be expected for regulatory proteins that control late gene expression. Bacteria were infected with wild-type SP01 and then pulse labelled at various times after infection. Crude extracts of the infected cells were then subjected to two-dimensional gel electrophoresis. Figure 2 indicates the proportion of total radioactivity that was incorporated into proteins V and VI during each pulse-labelling period. These results indicate that the synthesis of polypeptides V and VI was turned on synchronously at a middle time in the lytic cycle, after the appearance of polypeptide IV, the product of the early gene 28, but before the onset of late protein synthesis (not shown).

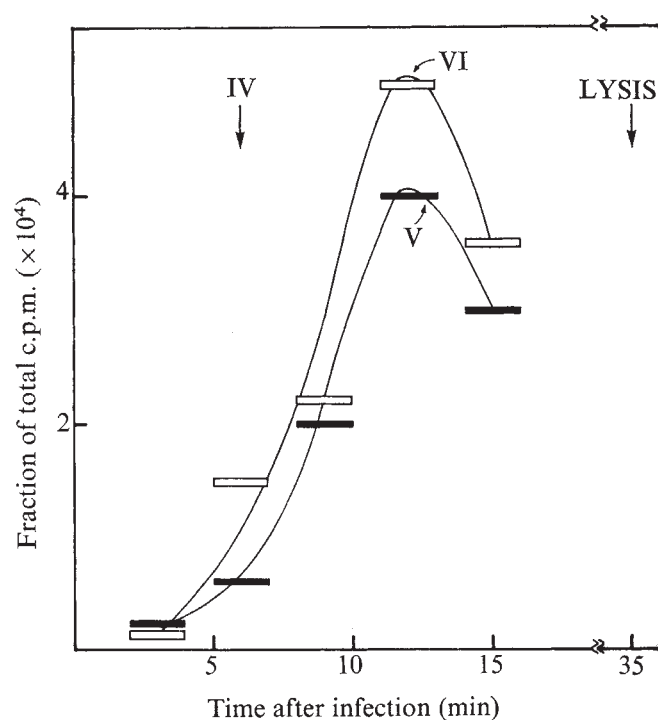


Fig. 2 Time course for the induction of subunits V and VI. Cultures of *su⁻* bacteria were infected with wild-type SP01, pulse labelled during the 2-min intervals indicated in the figure and chilled quickly. Crude extracts were prepared by sonication and ammonium sulphate precipitation as for purification of polymerase (Fig. 1). Samples of each extract ($\sim 8 \times 10^6$ c.p.m.) were diluted tenfold into "lysis buffer" and subjected to two-dimensional electrophoresis (Fig. 1). The gels were then autoradiographed to locate polypeptides IV, V and VI. Regions of the gel in the positions of V and VI were cut out, moistened and extracted in Protosol. The ordinate indicates the fraction of total radioactivity in each sample that was present in polypeptides V and VI. The peak time of subunit IV synthesis was determined by visual inspection of the autoradiograms.

(Two-dimensional electrophoresis did not resolve polypeptide IV sufficiently well to quantitate its time course of appearance. But the synthesis of this regulatory protein could be followed by visual inspection of the autoradiograms, and the arrow in Fig. 2 indicates the peak time of subunit IV synthesis.) The findings of Fig. 2 agree with a study¹⁰ that compared the time of appearance of polypeptides IV and V in association with RNA polymerase at different times in the lytic cycle.

Gene 34 codes for subunit V

To test directly whether subunit V is the protein product of gene 34, the gene 34⁻ nonsense mutant *susF4* was grown in two different suppressor (*su⁺*) strains of *B. subtilis* that carried the nonsense suppressors *sup-1*^{17,18} and *sup-3*^{19,20} in isogenic backgrounds. The *sup-1* and *sup-3* suppressors are thought to insert different amino acids¹² and a recent study in this laboratory⁴ indicated that the amino acid inserted by *sup-3* is more basic (or less acidic) than the residue inserted by *sup-1*. Thus, if subunit V is the product of gene 34, then polypeptide V generated by suppression of *susF4* with *sup-3* ought to be slightly more basic than the corresponding protein generated by suppression with *sup-1*. This strategy was previously used to assign subunit IV as the product of gene 28 (ref. 4).

To test the prediction that suppression would generate altered forms of polypeptide V, radioactively labelled RNA polymerase was separately purified by phosphocellulose chromatography from *susF4*-infected bacteria carrying either *sup-1* or *sup-3*. The phosphocellulose-purified

enzymes were then subjected to two-dimensional gel electrophoresis. A comparison of Fig. 3a and b suggests that polypeptide V from *sup-3* bacteria was slightly more basic than polypeptide V from the *sup-1* bacteria. Electrophoresis of a mixture of the phosphocellulose-purified enzymes from the two phage-infected suppressor-strains (Fig. 3c) confirmed that nonsense suppression had generated variants of polypeptides V but had not affected the isoelectric points of polypeptides IV and VI.

Although the two suppressor strains were constructed by transferring (by means of genetic transformation) the *sup-1* and *sup-3* suppressors into the same genetic background, it was still conceivable that the altered forms of polypeptide V were caused by some unknown difference between the suppressor strains other than the nonsense suppressors themselves. Therefore, to examine whether the generation of variants of polypeptide V was related directly to suppression of the *susF4* mutation, the isoelectric point of V produced by *susF4* in *sup-3* bacteria was compared with that of V produced by a spontaneous revertant of *susF4* in the same *sup-3* strain. Figure 3d and e shows that subunit V induced by the revertant was less basic than the corresponding polypeptide induced by the nonsense mutant. (Subunit V induced by the revertant in *sup-3* bacteria was indistinguishable from the corresponding protein generated by suppression of *susF4* in *sup-1* bacteria or the protein produced by wild-type SP01 in *su⁻* bacteria.)

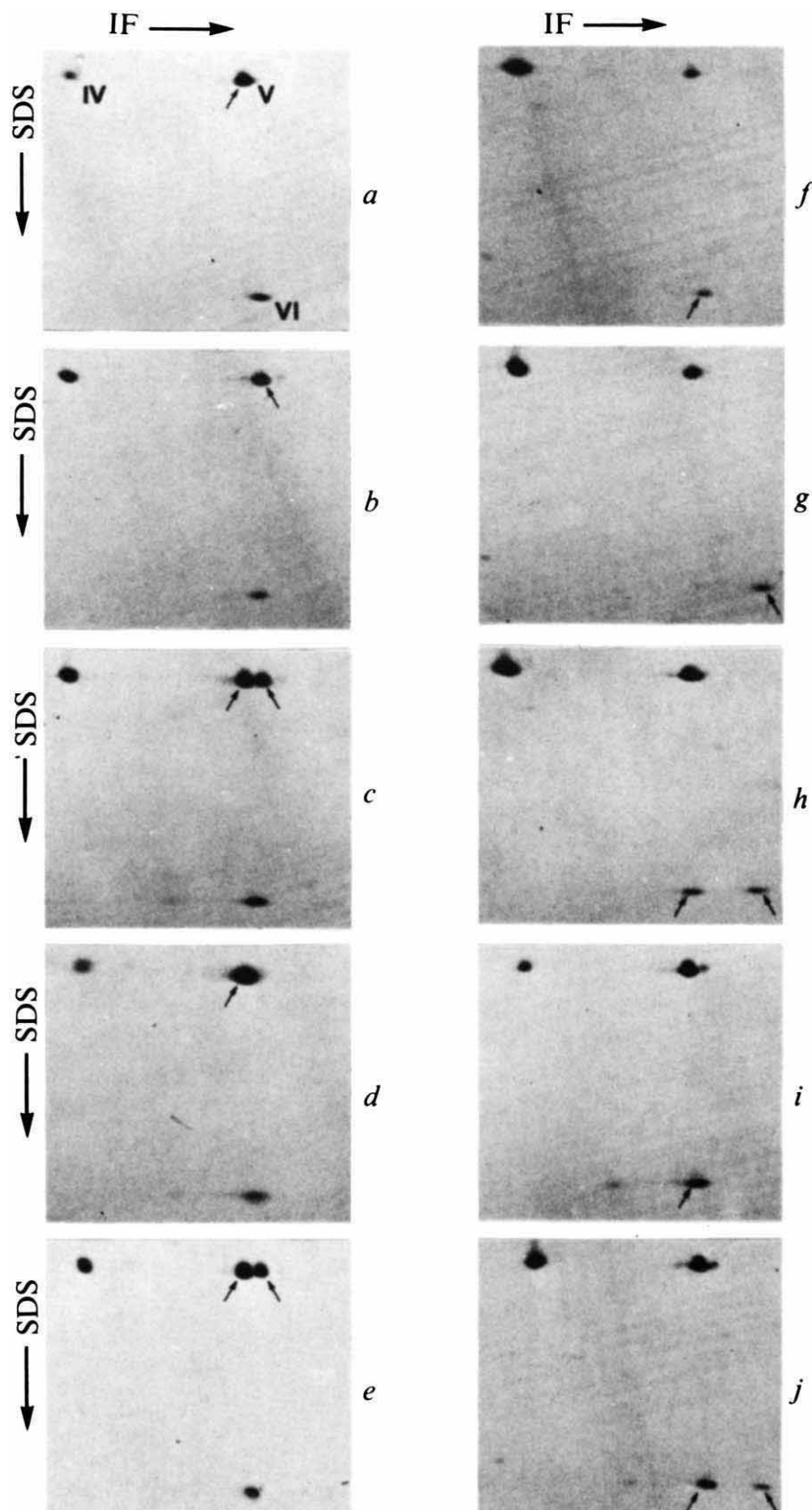
A possible complication in analysis of the isoelectric points of proteins is that charge changes in a single protein species can arise artefactually *in vitro* as a result of chemical modification, and cause the appearance of "multiple spots" in the isoelectric focusing dimension of the two-dimensional gels¹¹. To eliminate the possibility that the variants of subunit V had actually been produced by variable chemical modification *in vitro*, either during purification of polymerase or during two-dimensional analysis, the following 'double-label' mixing experiment was performed. RNA polymerase was purified from a mixture of *sup-1* and *sup-3*-carrying bacteria that had been separately labelled with ³H-methionine and ³⁵S-methionine, respectively, after infection with the mutant *susF4*. After two-dimensional electrophoresis, the distribution of each radioisotope in the isoelectric focusing dimension was determined for subunits IV, V and VI. While the distribution of both radioisotopes was identical for polypeptides IV and VI (Fig. 4a and c), the ³⁵S radioactivity in subunit V from the *sup-3* bacteria was in a more basic position than the ³H radioactivity from the *sup-1* strain (Fig. 4b). The reverse "double-label" mixing experiment (not shown), with ³⁵S-labelled *sup-1* bacteria and ³H-labelled *sup-3* bacteria, confirmed that the form of subunit V induced by *susF4* in *sup-3* cells was more basic than that induced by the mutant in the *sup-1* strain.

Gene 33 codes for subunit VI

If subunit VI is the product of gene 33, then suppression of the gene 33⁻ nonsense mutation, *susF14*, should generate altered forms of polypeptide VI without affecting the isoelectric point of subunits IV and V. The experiment shown in Fig. 3 confirms this prediction; *susF14* growing in *sup-3* bacteria produced a more basic form of VI (Fig. 3g) than that produced by *susF14* in *sup-1* bacteria (Fig. 3f and h) or that produced by a spontaneous revertant of *susF14* in *sup-3* bacteria (Fig. 3i and j) (or that produced by wild-type phage in *su⁻* bacteria).

Close examination of Fig. 3i and j reveals a faint spot of radioactivity on the acidic side of VI; this is probably attributable to charge heterogeneity introduced *in vitro* as referred to above. The "double-label" mixing experiment of Fig. 4 (as well as a corresponding experiment with the radioactive isotopes reversed) eliminates the possibility,

Fig. 3 Two-dimensional electrophoresis of RNA polymerase from *sup-1* and *sup-3* bacteria infected with SP01 nonsense mutants. Strains of *B. subtilis* carrying the nonsense suppressors *sup-1* and *sup-3* in isogenic backgrounds were constructed by transformation²¹ of the *su*⁻ strain Mu8u5u6 (Fig. 1) with DNA from the *sup-1* strain HA-101-B (ref. 15) and the *sup-3* strain *su*⁺3 (ref. 17). Suppressor-carrying transformants were isolated by selecting simultaneously for adenine and methionine prototrophy; the *purB6* and *metB5* mutations in Mu8u5u6 are suppressable markers^{12,19}. Purification and two-dimensional electrophoresis of RNA polymerase from phage-infected cells were as described in the legend to Fig. 1 except that the bacterial growth medium did not contain adenine. The autoradiograms are from electrophoreses of the following enzymes: *a*, RNA polymerase from *sup-1* bacteria infected with the mutant *susF4* (gene 34⁻); *b*, RNA polymerase from *sup-3* bacteria infected with the mutant *susF4* (gene 34⁻); *c*, a mixture of the enzymes from the experiments of *a* and *b*; *d*, RNA polymerase from *sup-3* bacteria infected with a spontaneous revertant of the mutant *susF4* (isolated by plating mutant phage on *su*⁻ bacteria); *e*, a mixture of the enzymes from the experiment of *b* and *d*; *f*, RNA polymerase from *sup-1* bacteria infected with the mutant *susF14* (gene 33⁻); *g*, RNA polymerase from *sup-3* bacteria infected with the mutant *susF14* (gene 33⁻); *h*, a mixture of the enzymes from the experiments of *f* and *g*; *i*, RNA polymerase from *sup-3* bacteria infected with a spontaneous revertant of the mutant *susF14* (isolated by plating mutant phage on *su*⁻ bacteria); *j*, a mixture of the enzymes from the experiments of *g* and *i*.



however, that the difference in charge of the *sup-1* and *sup-3*-generated variants of polypeptide VI could have been caused completely by variable chemical modification introduced *in vitro*. Figure 4 also confirms that suppression of the gene 33⁻ nonsense mutant had specifically affected the isoelectric point of VI (Fig. 4f) without affecting subunits IV (Fig. 4d) or V (Fig. 4e).

Scheme for regulation

The finding that altered forms of polypeptides V and VI can be generated independently by suppression of nonsense mutations in SP01 genes 34 and 33 strongly suggests that these phage-induced subunits of RNA polymerase are coded by the regulatory cistrons that control late gene expression. A complicated alternative interpretation is that the SP01

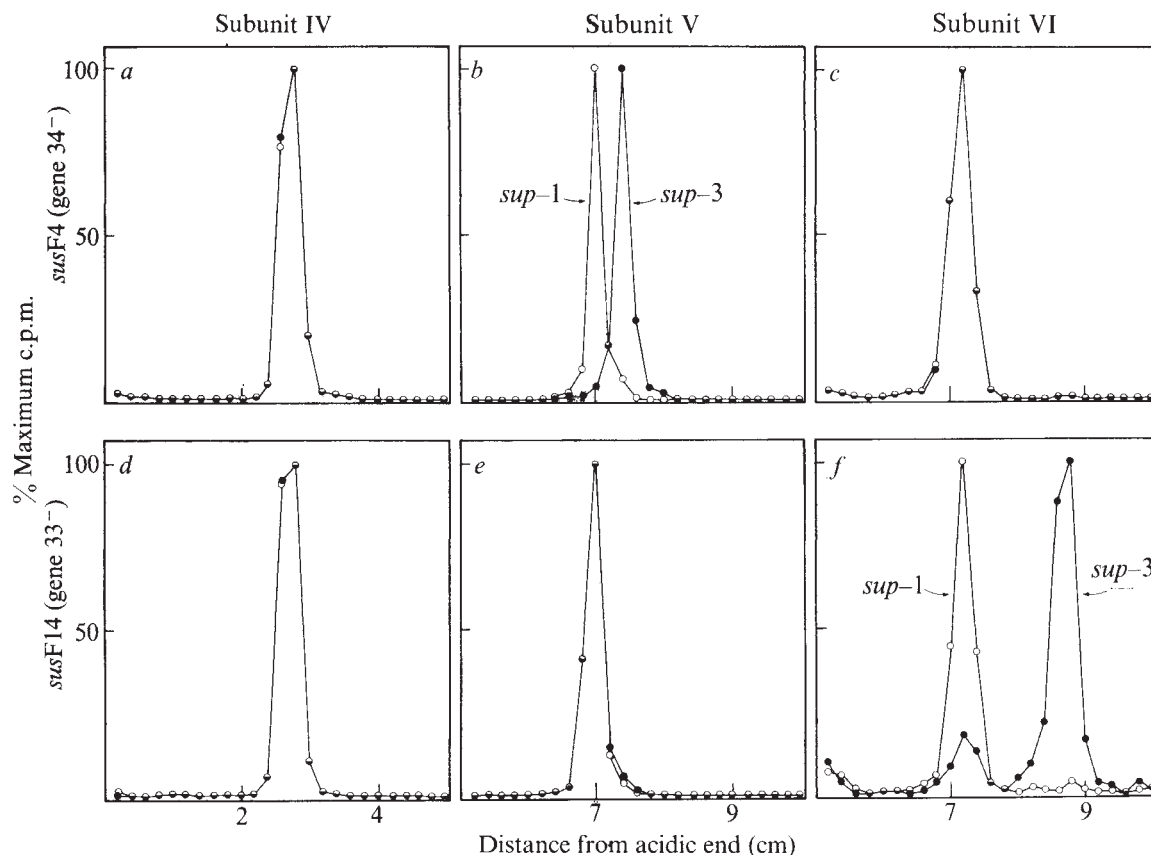


Fig. 4 Isoelectric focusing of subunits IV, V and VI from mixtures of *sup-1* and *sup-3* bacteria separately labelled with different radioisotopes after phage-infection. Cultures of *sup-1* and *sup-3* bacteria were labelled separately with ^3H -methionine (Schwarz/Mann) and ^{35}S -methionine respectively, after infection with the nonsense mutant indicated below. RNA polymerase was then purified from a mixture of the infected cells and subjected to two-dimensional electrophoresis as described in the legend to Fig. 1. After autoradiography to locate the position of subunits IV, V and VI, strips were cut from the gels along the isoelectric

focusing dimension at the positions of IV, V and VI in the SDS dimension. Each strip was then cut into 2-mm slices and the amount of ^3H ($\circ$) and ^{35}S ($\bullet$) in each slice was measured in a scintillation counter after moistening and extracting the slices in Protosol. *a-f* show the distribution of each radioisotope along the IF dimension in *a*, subunit IV from *susF4* infected cells; *b*, subunit V from *susF4*-infected cells; *c*, subunit VI from *susF4*-infected cells; *d*, subunit IV from *susF14*-infected cells; *e*, subunit V from *susF14*-infected cells; *f*, subunit VI from *susF14*-infected cells.

regulatory genes actually code for enzymes that chemically modify SP01-induced subunits of RNA polymerase *in vivo*, and that modifications by the *sup-3*-suppressed gene products somehow generate more basic (or less acidic) polymerase subunits than modifications by the *sup-1* suppressed gene products. This interpretation, however, seems improbable, especially in the light of the fact that isoelectric point variants of three different proteins can be generated independently by suppression of nonsense mutations in three different SP01 genes (genes 33 and 34, Figs 3 and 4; gene 28, ref. 4).

If these gene-product assignments are correct, why does

the *susF14* mutation in gene 33 prevent the normal synthesis of the gene 34 protein (Fig. 1)? Since genes 33 and 34 are tightly linked on the genetic map of SP01 (ref. 3), one possible explanation is that the regulatory cistrons that control late transcription are located in the same operon and that the *susF14* nonsense mutation exerts a "polar" effect on the expression of gene 34. But since the *susF4* and *susF14* mutants complement each other (albeit weakly)³, this explanation demands that the *susF14* mutant actually synthesises a small amount of the gene 34 protein (smaller than can be detected in Fig. 1) and that this small quantity is sufficient to promote late gene expression. Another possible explanation of the pleiotropic effect of the *susF14* mutation is that the expression of gene 34 is controlled directly by the gene 33 protein. This possibility is not necessarily inconsistent with the time course experiment of Fig. 2, or with the finding that the genes 33 and 34 proteins act synergistically to alter the global pattern of SP01 transcription *in vitro*⁹.

In conjunction with the previous finding that regulatory gene 28 codes for a phage-induced subunit of RNA polymerase⁴, the further gene-product assignments reported here suggest the following scheme for the temporal control of phage SP01 gene expression (Fig. 5). Immediately on infection, host sigma factor directs the transcription of phage early genes. Next, at an intermediate time in the lytic cycle the gene 28 protein (itself the product of an early gene) turns on the expression of middle genes by

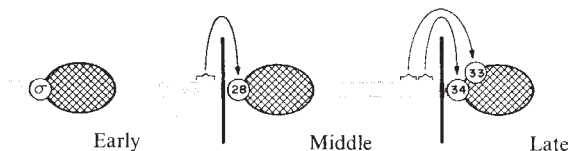


Fig. 5 Proposed model for the temporal control of phage SP01 gene expression. The figure illustrates schematically the three major categories of SP01 gene transcription; the figure is not intended to represent the actual physical arrangement of early, middle and late genes on the phage genome or the number and arrangement of promoter sites. The cross-hatched ellipses represent the host subunits of RNA polymerase other than sigma factor.

binding to the host polymerase in the place of sigma factor. Finally, the gene 33 and 34 proteins (the products of middle genes) bind to the host transcriptase and activate late gene expression. This scheme is undoubtedly an oversimplification since it does not account for the turn off of subclasses of the early and middle genes at different times in the lytic cycle (see ref. 1). In analogy with the model of Fig. 5, the coliphage T4 regulatory genes that control late gene expression code for proteins that bind to the host RNA polymerase²²⁻²⁴.

I thank J. Pero and R. Losick for helpful discussions, A. L. Sonenshein for assistance with genetic transformations and R. Losick for help with the manuscript. This work was supported by a grant from the NSF.

Received April 26; accepted July 7, 1976.

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Bacteriophage SP01 regulatory proteins directing late gene transcription *in vitro*

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Expression of the temporally defined "late" genes of Bacillus subtilis phage SP01 requires the protein products of regulatory genes 33 and 34. These proteins have now been isolated in pure form. Together with a host factor delta, they act synergistically to direct "late" gene transcription in vitro by core RNA polymerase.

THE temporal programme of gene expression by *Bacillus subtilis* phage SP01 is controlled by phage-coded regulatory proteins that interact with the bacterial RNA polymerase (for a review see ref. 1). Phage middle-gene transcription is activated at 4-5 min into the lytic cycle by the SP01 gene 28 product^{2,3}, a regulatory protein that binds to *B. subtilis* RNA polymerase^{4,5}. Enzyme containing the gene 28 protein asymmetrically transcribes phage middle genes *in vitro*⁶⁻⁹. Phage late-gene transcription is turned on 8-12 min after infection and this RNA synthesis seems to require the products of two SP01 regulatory genes called 33 and 34 (refs 2 and 3). As Fox shows in an accompanying article¹⁰, genes 33 and 34 code for phage-induced subunits of the bacterial RNA polymerase, and Pero *et al.*⁷ have shown that enzyme containing these phage-coded polypeptides preferentially synthesises late RNA *in vitro*. Pero *et al.*^{6,7} have also identified a *B. subtilis* protein called δ that is associated with RNA polymerase from both uninfected and infected bacteria, and that markedly enhances the specificity of transcription by the phage-modified forms of RNA polymerase that copy middle and late genes *in vitro*.

To determine whether the subunits of RNA polymerase coded by genes 33 and 34 are directly responsible for the synthesis of late RNA *in vitro*, we have isolated these phage gene products in pure form free of the host subunits of RNA polymerase. By a reconstitution experiment we have shown that the gene 33 protein, the gene 34 protein and host δ protein act cooperatively to activate late gene transcription by core RNA polymerase.

Isolation of phage regulatory proteins

Highly purified RNA polymerase containing the protein products of genes 33 and 34 (formerly called subunits VI and V, respectively^{6,7}) was isolated from phage SP01-infected cells of *B. subtilis* collected late in the lytic cycle (see legend to Fig. 1). The phage regulatory proteins were dissociated from core enzyme by denaturation in 6 M urea. To separate the proteins from the host subunits of polymerase, the denatured enzyme complex was applied to a phosphocellulose column equilibrated with buffer containing 6 M urea, and eluted with a linear gradient of KCl. The phage regulatory proteins were located by subjecting alternate fractions from the gradient elution to sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis (Fig. 1a). Next, fractions 15-19 and 31-33 containing the phage regulatory proteins and some contaminating host polymerase subunits were subjected to gel filtration on Sephadex G-100 as a final purification step. SDS slab gel analysis showed that gel filtration had separated the proteins from each other and from the subunits of core polymerase (Fig. 1b). It is interesting that the phage proteins which have molecular weights of 24,000 and 13,500 (ref. 6), respectively, eluted from the Sephadex column at positions corresponding to ovalbumin (molecular weight 43,000) and to chymotrypsinogen (25,700). This could indicate either that the regulatory proteins have an unusual shape or that they form dimers in 6 M urea.

Purified gene 33 and gene 34 proteins were obtained by pooling fractions 40 to 46, and fractions 32 to 34, respectively, from the gel filtration column. Figure 2 shows that each pooled fraction apparently contained only a single highly purified polypeptide (Fig. 2b and c), corresponding in size to the regulatory proteins in phage-modified RNA polymerase (Fig. 2a). As further evidence for the identification of the purified regulatory proteins, a mixing experiment showed that the purified phage proteins of Fig. 2 comigrated during SDS gel electrophoresis with the radioactively-labelled protein

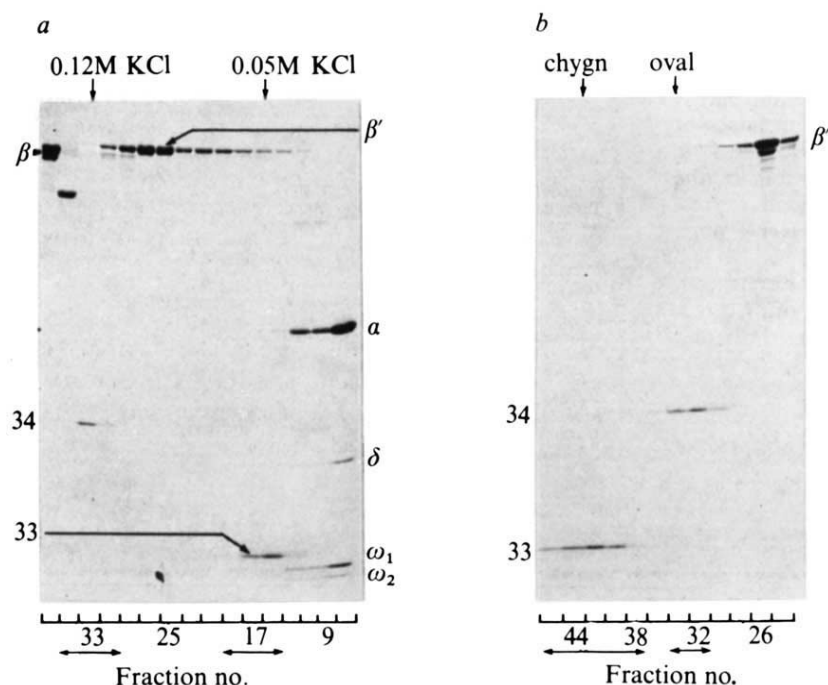
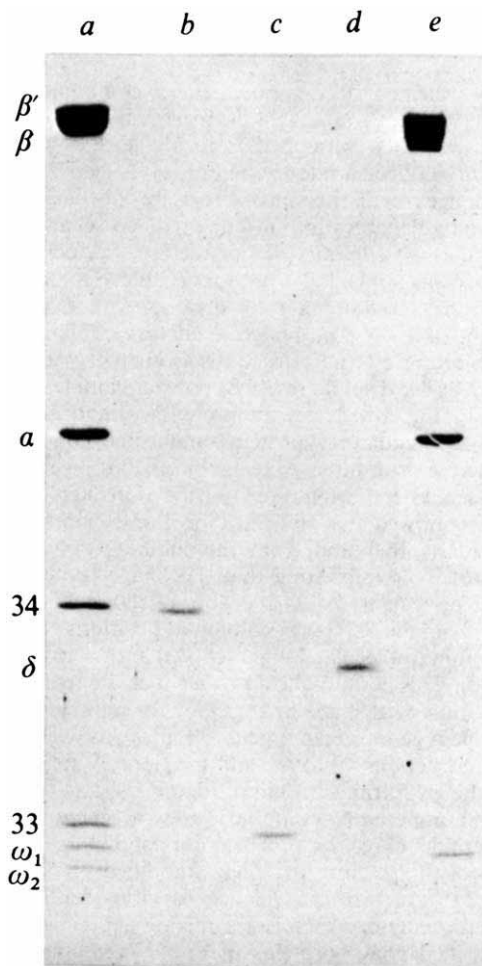


Fig. 1 Purification of the protein products of genes 33 and 34 by phosphocellulose chromatography and gel filtration in denaturing conditions. *B. subtilis* was infected with SP01 at a multiplicity of infection of 5 and collected 22 min after infection. RNA polymerase was purified from 80 g of infected cells through DEAE-Sephadex chromatography as described previously¹¹ except that the 2 M NaCl extraction during the phase partitioning step was omitted. One sixth of the DEAE-Sephadex-purified enzyme (5 mg), containing the subunits of core RNA polymerase⁸ (β' , β , α , ω_1 and ω_2), δ protein, and the polypeptide products of genes 33 and 34, was denatured by dialysis against buffer D (6 M urea, 0.05 M Tris, pH 8.0, 0.1 mM EDTA, 0.4 mM DTT and 0.05 M KCl). *a*, Denatured enzyme was then applied to a phosphocellulose column (2 cm $\times$ 8 cm) equilibrated with buffer D and eluted with a linear gradient of 0.05–0.33 M KCl in buffer D. Fractions (3 ml) were collected and samples (25 μ l) from every other fraction were subjected to electrophoresis on 25-cm-long slab gel containing SDS in a Tris-glycine buffer and a linear gradient of 7–15% acrylamide¹². *b*, Fractions 15–19 and 31–33 containing the protein products of genes 33 and 34 were pooled, concentrated against Ficoll (Sigma) to a final volume of 1.5 ml, dialysed against buffer D for 2 h at 4 °C and then applied to a Sephadex G-100 column (0.9 cm $\times$ 100 cm) equilibrated with buffer D. Fractions (1 ml) were collected and samples (50 μ l) from every other fraction were subjected to SDS slab

gel electrophoresis. To renature the phage-coded proteins, gene 34 protein (in the pooled fractions 32–34) and gene 33 protein (in the pooled fractions 40–46) were mixed separately with core RNA polymerase (300 μ g) from uninfected cells, concentrated threefold against Ficoll and then dialysed against the renaturation buffer (0.05 M Tris, pH 8.0, 0.1 mM EDTA, 25% glycerol, 8 mM DTT and 0.3 M KCl) for 3–6 h at 4 °C (ref. 13). The added core RNA polymerase which was inactivated on mixing with the regulatory proteins in buffer D did not regain significant activity (less than 0.2%) during renaturation. Thus the renatured regulatory protein fractions contained inactive core enzyme. The renatured regulatory proteins were stored at –20 °C until used in the reconstitution experiments. We were guided in the isolation and renaturation of the SP01-coded regulatory proteins by a discussion of the reconstitution of *E. coli* RNA polymerase by Zillig *et al.*¹³.



products of genes 33 and 34 described in Fig. 1a of the accompanying article¹⁰ (date not shown).

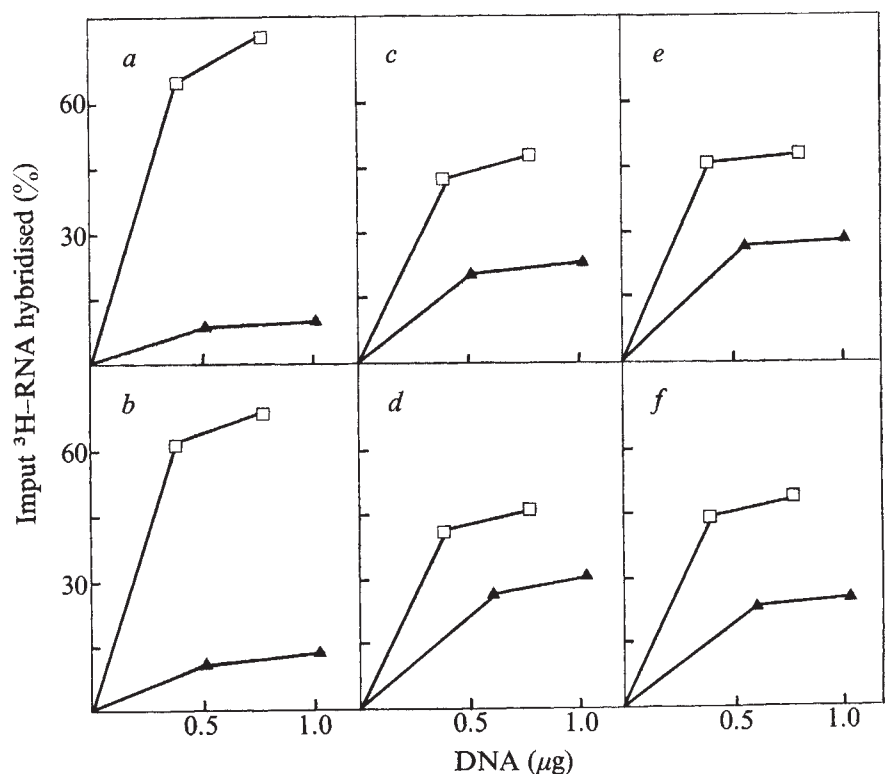
Finally, the two proteins which had been purified in the presence of 6 M urea were renatured in the presence of core RNA polymerase from uninfected cells (see legend to Fig. 1 for experimental details). In the conditions we used the presence of core enzyme was essential for recovery of the phage regulatory proteins in active form.

Strand selectivity of transcription

As a test of whether the renatured phage regulatory proteins would direct specific RNA synthesis, we asked whether these phage-coded polypeptides would cause selective transcription from the heavy (H) strand of native SP01 DNA, the strand from which late RNA is copied *in vivo*^{6,14}. Figure 3b shows that in the presence of both phage proteins and host δ protein, purified core enzyme preferentially transcribed strand H of SP01 DNA (an H/L hybridisation ratio of 5.3). For comparison, core enzyme containing only added δ protein exhibited only a small preference for strand H (an H/L ratio of 1.9, Fig. 3f) while the original phage-modified polymerase plus added δ exhibited a strong preference for H strand DNA (an H/L

Fig. 2 SDS-polyacrylamide slab gel electrophoresis of RNA polymerase subunits from uninfected and SP01-infected *B. subtilis*. RNA polymerase containing the protein products of genes 33 and 34 was purified as described previously (enzyme C in ref. 6). Purification of the regulatory protein products of genes 33 and 34 is described in the legend to Fig. 1. Delta was isolated from uninfected cells as previously described⁷ except that a DEAE-Sephadex column was used instead of a poly(C)-cellulose column to separate the δ subunit of RNA polymerase from the σ subunit; δ eluted at 0.5 M KCl while σ eluted at 0.25 M KCl. Purification of core RNA polymerase from uninfected cells has been described previously¹¹. The purified proteins were subjected to electrophoresis as described in the legend to Fig. 1. *a*, RNA polymerase containing the protein products of genes 33 and 34; *b*, gene 34 protein (fractions 32–34 from Fig. 1b); *c*, gene 33 protein (fractions 40–46 from Fig. 1b); *d*, δ protein; *e*, core RNA polymerase.

Fig. 3 Effect of phage regulatory proteins and host protein on strand selective transcription. Core RNA polymerase (13 μ g) from uninfected cells (Fig. 2e) was mixed with the indicated combination of the following: 1.1 μ g of renatured gene 33 protein, 1.5 μ g of renatured gene 34 protein, 2.0 μ g of δ protein, or mock renaturation reaction mixture to which no regulatory protein had been added. 3 H-RNA was synthesised *in vitro* as described previously⁶ except that the reaction mixtures (0.5 ml) contained 0.025 M KCl, 0.9 mg anti-sigma gamma globulin¹⁵ and were incubated at 32 °C for 30 min. (The anti-sigma gamma globulin was necessary to inactivate traces of σ that contaminated the core RNA polymerase and that severely interfered with the transcriptional specificity of the reconstituted enzymes.) Hybridisation of RNA and preparation of DNA strands were as described previously⁶. Hybridisation reactions contained the indicated amounts of H strand ($\square$) and L strand ($\blacktriangle$) SP01 DNA and the following 3 H-RNAs (representing about 5% of the product of the *in vitro* RNA synthesis reactions) in 125 μ l of $2 \times$ SSC. *a*, RNA synthesised by RNA polymerase (6.0 μ g) containing the protein products of genes 33 and 34 together with host δ purified as described in the legend to Fig. 1 (input 8,960 c.p.m.; background 490 c.p.m.). *b*, RNA synthesised by core polymerase+renatured gene 33 protein+renatured gene 34 protein+ δ protein (input 9,180 c.p.m.; background 250 c.p.m.). *c*, RNA synthesised by core polymerase+renatured gene 33 protein+ δ protein (input 10,510 c.p.m.; background 250 c.p.m.). *d*, RNA synthesised by core polymerase+renatured gene 34 protein+ δ protein (input 7,250 c.p.m.; background 130 c.p.m.). *e*, RNA synthesised by core polymerase+renatured gene 33 protein+renatured gene 34 protein (input 15,830 c.p.m.; background 340 c.p.m.). *f*, RNA synthesised by core polymerase+ δ protein+mock renaturation reaction mixture (input 7,010 c.p.m.; background 200 c.p.m.).



ratio of 8.4, Fig. 3a). Specific RNA synthesis by the reconstituted enzyme required both the protein products of genes 33 and 34, for the strand selectivity of transcription in the absence of either regulatory protein (H/L ratio of 2.0 and 1.8, Fig. 3c and d) was not measurably higher than in the absence of both regulatory gene products (H/L = 1.9, Fig. 3f). Also, as expected from our previous findings⁷, specific transcription by reconstituted enzyme required host δ protein in addition to the phage regulatory proteins; the strand selectivity of transcription by reconstituted enzyme lacking added δ (an H/L ratio of 1.9, Fig. 3e) was only slightly higher than that for core enzyme alone (an H/L of 1.3, ref. 6 and data not shown). Thus, the reconstitution of enzyme directing strand selective transcription required the addition of both regulatory proteins and host δ factor to core polymerase.

As an independent test of the asymmetry of transcription we measured the formation of RNA-RNA duplexes between labelled RNA synthesised *in vitro* and unlabelled early, middle and late RNAs isolated from phage-infected cells. The percentage of 3 H-RNA that annealed to the *in vivo* synthesised messenger RNAs was taken as a measure of the proportion of *in vitro* generated RNA representing anti-messenger sequences. As expected, a large percentage of the RNA synthesised by core RNA polymerase contained anti-mRNA sequences (line 1, Table 1). Similarly, RNA synthesised by partially reconstituted enzyme containing core polymerase and host δ but lacking the products of either gene 34 (line 2, Table 1) or gene 33 (line 3, Table 1) contained high levels of anti-mRNA. Likewise, if both phage gene products were included but host δ factor was omitted, a large proportion of the *in vitro* synthesised RNA contained anti-messenger sequences (line 4, Table 1). In contrast, very little anti-mRNA was generated by the fully reconstituted enzyme containing both phage-coded regulatory proteins and host δ factor (line 5, Table 1). For comparison, core polymerase plus host σ factor also synthesised low levels of anti-messenger (line 6, Table 1). Thus, the genes 33 and 34 proteins and host δ protein act

cooperatively to catalyse asymmetric transcription of native SP01 DNA.

Synthesis of late RNA

Hybridisation-competition experiments indicated that core enzyme that had been reconstituted with the protein products of genes 33 and 34 and host δ factor preferentially synthesised

Table 1 Anti-mRNA synthesised *in vitro*

Enzyme	% 3 H-RNA forming duplexes with <i>in vivo</i> RNAs		
	Early RNA	Middle RNA	Late RNA
Core polymerase	32	33	44
Core polymerase+ δ +gene 33 protein	31	30	40
Core polymerase+ δ +gene 34 protein	32	31	43
Core polymerase+gene 33 protein+gene 34 protein	41	34	34
Core polymerase+ δ +gene 33 protein+gene 34 protein	12	9	13
Core polymerase+ σ	6	7	15

RNA synthesis *in vitro* was as described in the legend to Fig. 3 except that no anti-sigma gamma globulin was included in the experiment of line 6. Early RNA was isolated from bacteria treated with chloramphenicol (150 μ g ml⁻¹) before infection; middle RNA was isolated from bacteria collected 25 min after infection by a mutant phage (*susF4*) blocked in the synthesis of late RNA²; and late RNA was isolated from cells collected 25 min after infection by wild-type SP01. Unlabelled RNA was prepared as previously described⁷. The RNA-RNA annealing reactions contained about 10 ng of 3 H-RNA (800 c.p.m. ng⁻¹) synthesised *in vitro* by the indicated enzyme and about 0.15 mg of the indicated unlabelled RNA in a total volume of 150 μ l of $2 \times$ SSC. The reactions were incubated at 70 °C for 3 h, chilled and incubated with RNase A (20 μ g) and RNase T1 (20 U) in 1 ml of $2 \times$ SSC for 1 h at 35 °C. RNase resistant material was precipitated with 5% trichloroacetic acid.

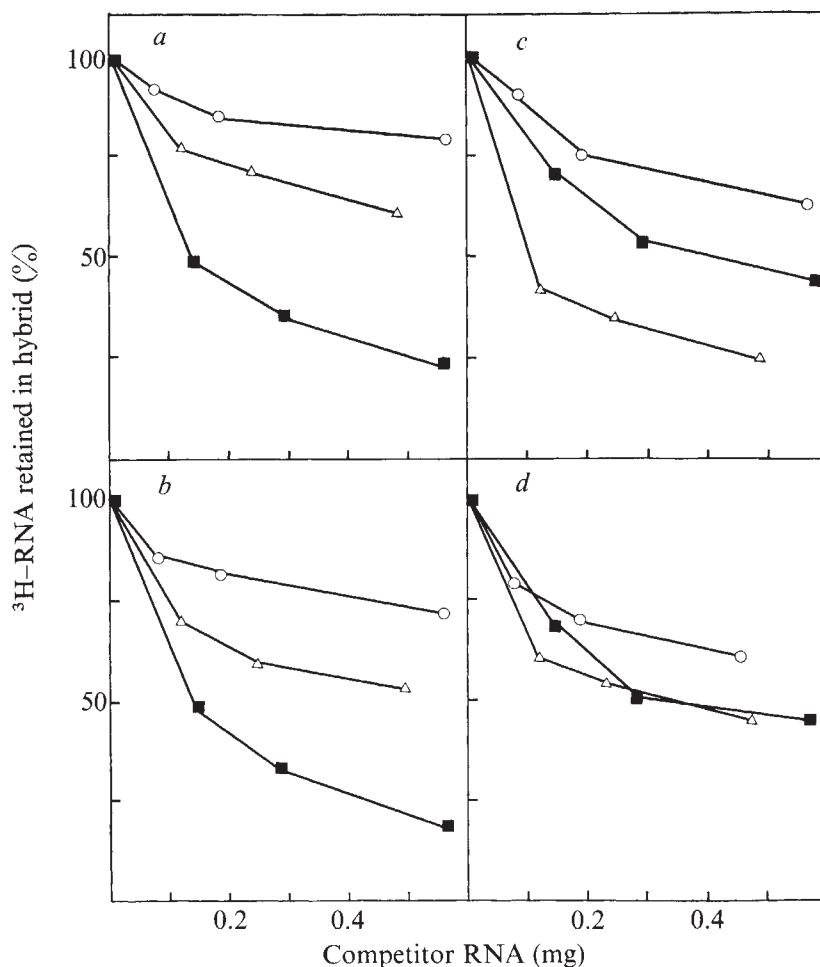


Fig. 4 Hybridisation competition of *in vitro* synthesised ³H-RNAs. RNA was synthesised and hybridisations were performed as described in the legend to Fig. 3 except that the hybridisation reaction was incubated for 3 h at 68 °C. Hybridisation reactions contained 2.5 µg of denatured SP01 DNA, the indicated amounts of unlabelled early (○), middle (△) or late (■) competitor RNAs prepared as described in the legend to Table 1, and the following, *in vitro* synthesised ³H-RNA (representing about 5% of the product in the reactions listed below) in a total volume of 200 µl of 2×SSC. *a*, RNA synthesised by RNA polymerase (6.0 µg) containing the protein products of genes 33 and 34 together with host δ; 50% of the input RNA (8,440 c.p.m.) hybridised in the absence of competitor. *b*, RNA synthesised by core polymerase (13 µg)+renatured gene 33 protein (1.0 µg)+renatured gene 34 protein (1.5 µg)+δ protein (2.0 µg); 51% of the input RNA (9,570) hybridised in the absence of competitor. *c*, RNA synthesised by RNA polymerase (10 µg) containing the gene 28 protein (enzyme B of ref. 7)+δ protein (1.2 µg); 50% of the input RNA (9,600 c.p.m.) hybridised in the absence of competitor. *d*, RNA synthesised by core polymerase (13 µg)+δ (2.0 µg)+mock renaturation reaction mixture; 52% of the input RNA (7,150 c.p.m.) hybridised in the absence of competitor. For each hybridisation a background of less than 7% of the input radioactivity was subtracted.

late RNA sequences. ³H-RNA synthesised *in vitro* by reconstituted enzyme was annealed to denatured SP01 DNA in the presence of unlabelled competitor RNAs containing predominantly early, middle or late classes of RNA. To be sure that the middle RNA did not contain late sequences, this competitor was isolated from bacteria that had been infected with a mutant blocked in late gene expression (see legend to Table 1). (Similar competition results, however, were obtained with competitor RNA isolated at a middle time after infection with wild-type SP01 (unpublished results and ref. 7).) Figure 4b shows that the hybridisation of RNA synthesised *in vitro* by the reconstituted enzyme was more effectively inhibited by the late RNA competitor than by the middle or early RNA competitors. This pattern of competition was similar to that obtained for the hybridisation of RNA synthesised by the original phage-modified polymerase containing the protein products of genes 33 and 34 together with δ protein (Fig. 4a). In contrast, the hybridisation of RNA synthesised *in vitro* by polymerase containing the gene 28 protein plus δ factor was more effectively inhibited by middle RNA competitor than by late or early RNA competitors (Fig. 4c). As a control, the hybridisation of the symmetrical RNA generated by core polymerase plus δ was not preferentially inhibited by the late RNA competitor (Fig. 4d). Also, analogous hybridisation experiments indicated that partially reconstituted enzymes lacking either the gene 33 product, the gene 34 product or δ protein did not preferentially generate late RNA sequences. Thus, like the strand selectivity of transcription, the preferential transcription of SP01 late genes *in vitro* required the combined effects of both phage-coded regulatory proteins and host δ factor.

Gene products control transcription

Our reconstitution experiments establish that the products of genes 33 and 34 are directly responsible for promoting the

asymmetrical transcription of phage SP01 late genes *in vitro*. Since the protein products of genes 33 and 34 were isolated in pure form, we further conclude that these two regulatory proteins are apparently the only SP01-coded proteins required for the synthesis of late RNA *in vitro*. We have not, however, determined whether these two proteins are sufficient to direct the transcription of all SP01 late genes or only a subset of phage late sequences. As expected from an earlier study⁷, the reconstitution experiments also show that the specific transcription of phage late genes required host protein δ but not the σ subunit of RNA polymerase.

The synergistic effect of the protein products of genes 33 and 34 on late gene transcription *in vitro* is in apparent agreement with earlier genetic studies indicating that a mutation in either gene 33 or gene 34 alone is sufficient to block late RNA synthesis *in vivo*^{2,3}. The genetic experiments do not, however, necessarily predict that both regulatory proteins are simultaneously required for the expression of late genes *in vivo*. For example, as discussed by Fox¹⁰, it is possible, though unlikely, that the gene 33 protein serves only to activate regulatory gene 34 and that the gene 34 protein alone is sufficient to direct late RNA synthesis *in vivo*. The *in vitro* requirement for both regulatory proteins for asymmetric transcription of late genes casts doubt on such a model.

It is likely that the SP01 gene 28 protein also interacts with *B. subtilis* RNA polymerase to activate middle-gene expression. Fox *et al.*⁴ have shown that gene 28 codes for a phage-induced subunit of polymerase, and Pero *et al.*^{6,7} and Duffy and Geiduschek⁶ have shown that RNA polymerase containing the gene 28 product asymmetrically transcribes phage middle genes *in vitro*. In further support for the idea that a phage-coded protein confers specificity for middle genes on RNA polymerase, Duffy *et al.*¹⁶ have partially purified a fraction from crude extracts of SP01-infected cells that causes preferential

synthesis of middle RNA by core enzyme. This fraction contained a phage-induced protein similar in size to the gene 28 product that was able to bind to core RNA polymerase from uninfected cells. Petrusek *et al.*¹⁷ have attempted to isolate this transcriptional determinant by denaturing in urea an RNA polymerase fraction containing several phage-induced proteins, including polypeptides that apparently correspond to the products of genes 28, 33 and 34. Unfortunately, in both cases the protein presumed to direct middle-gene transcription was only one component of a partially purified fraction containing several proteins and it was not excluded that another polypeptide had been responsible for conferring transcriptional specificity on the core enzyme. Indeed, a new protein species has been identified that is apparently distinct from the product of gene 28 and that promotes asymmetric transcription of phage middle genes *in vitro*¹⁸. Proof that the gene 28 protein itself directly influences the transcriptional specificity of RNA polymerase therefore awaits the isolation of this phage-coded regulatory protein in pure form.

In summary, our findings establish that two phage-coded subunits of RNA polymerase are directly responsible for promoting specific gene transcription. How do the protein products of genes 33 and 34 work? As they influence transcription in the absence of the host σ factor, we suppose that they cause RNA polymerase to initiate transcription at specific promoter sites for phage late genes. A test of this idea now requires the isolation of fragments of SP01 DNA specifying individual late gene transcripts.

We thank Jane Nelson for preparing the DNA strands and competitor RNAs, R. Losick, T. Fox and W. Gilbert for helpful discussions, and R. Losick for help with the manuscript. This work was supported by a grant to J.P. and R. Losick from the NSF. R.T. is a junior fellow of the Harvard Society of Fellows.

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Nonsense suppressors of *Saccharomyces cerevisiae* can be generated by mutation of the tyrosine tRNA anticodon

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The yeast amber suppressor, SUP5-a, was previously shown to cause the insertion of tyrosine at the sites of UAG nonsense codons. Nucleotide sequencing established that this SUP5-a suppressor specifies a mutant tyrosine transfer RNA (tRNA) which has the anticodon CΨA instead of the normal GΨA, an alteration identical to that found in the tyrosine-inserting amber suppressor from Escherichia coli.

THE three codons UAG (amber), UAA (ochre) and UGA (opal) serve as signals for polypeptide chain termination during

messenger RNA translation in various prokaryotic and eukaryotic organisms^{1–9}. When one of these codons arises internally in a structural gene as a result of mutation, the gene product is an incomplete polypeptide. Such nonsense mutations can be reverted by an unlinked suppressor mutation, which alters one of the components required for protein synthesis and facilitates the formation of a completed polypeptide through the insertion of an amino acid at the position of the nonsense codon. The most extensively studied suppressors arise by mutation of the structural genes for tRNA although other suppressors can arise by mutation of genes controlling tRNA modification enzymes or ribosomal proteins (for review see refs 10–12). Sequencing of suppressor tRNAs has shown that the base changes giving rise to the suppressor function can occur at the anticodon^{13–16} and in other regions of the tRNA structure¹⁷.

The eukaryotic yeast *Saccharomyces cerevisiae* can form

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nonsense suppressors similar to those of *E. coli* (for review see ref. 18). Several of these nonsense suppressors have been characterised by their action on *iso-1*-cytochrome *c* mutants which contain defined ochre (UAA) and amber (UAG) mutations^{19,20}. In contrast to any of the *E. coli* suppressors, a major class of suppressors from yeast acts specifically on UAA and not UAG codons. Whereas bacterial ochre suppressors recognise both UAA and UAG codons, suppressors acting specifically on only UAG codons are found in both *E. coli* and yeast. The amino acids inserted by the yeast suppressors have been determined from protein analysis of *iso-1*-cytochrome *c* in suppressed mutants. So far tyrosine, serine and leucine-inserting suppressors have been identified (refs 21–25, 45). Studies of translation *in vitro* of mRNA containing nonsense codons have shown that the suppressor activities of at least some of the yeast suppressors are due to altered tRNAs^{26,27}. Such yeast suppressor tRNAs may have a potential application in methods for the selection and biochemical characterisation^{28,46} of nonsense mutants of mammalian cells and their viruses. But although the *in vitro* studies showed that suppression is mediated by tRNAs, the results did not indicate whether the suppressor mutations caused alterations in either the nucleotide sequences of tRNAs or the enzymes that modify them. Also the tRNA giving rise to a suppressor cannot always be identified from the amino acid inserted at the nonsense codon. This is true in the case of the recessive-lethal glutamine and tryptophan-inserting Su 7 suppressor of *E. coli*, which is derived from tryptophan tRNA by a single base substitution in the anticodon^{16,29,30} as well as for the glutamine-mischarging mutants derived from *E. coli*, Su $\dagger$ tRNA^{Tyr} (ref. 34).

We present here direct evidence that at least one yeast suppressor arose by the mutational alteration of the nucleotide sequence of a tRNA, and we report the nucleotide change in this suppressor tRNA. The suppressor examined in this study, *SUP5-a*, is typical of one of the eight distinct suppressors (*SUP2-a*, *SUP3-a*, *SUP4-a*, *SUP5-a*, *SUP6-a*, *SUP7-a*, *SUP8-a* and *SUP11-a*) that cause insertion of tyrosine at UAG

sites^{22,24}. In addition, a parallel series of allelic suppressors, *SUP2-o*, *SUP3-o*, and so on, causes the insertion of tyrosine at UAA sites²¹. Nucleotide sequencing established that *SUP5-a* determines a mutant form of tyrosine tRNA which has the anticodon C Ψ A instead of the normal G Ψ A. This altered anticodon was observed whether the amber suppressor arose from the wild-type gene or from the UAA suppressor *SUP5-o*. With our techniques we were unable to reveal the tRNA alteration associated with the *SUP5-o* suppressor. We were also unable to detect altered tRNA when the *SUP5-a* suppressor mutated to the less efficient *SUP5-a* $\rightarrow$ *a*¹ suppressor³².

Minor tRNA^{Tyr} with altered anticodon in amber-suppressing strains

Only one tyrosine tRNA is found in *S. cerevisiae* and its nucleotide sequence is known³³. If the tyrosine-inserting suppressors of this organism are the products of single-base substitutions of tRNA^{Tyr} genes, such a suppressor tyrosine tRNA would be expected to copurify with the bulk of the tyrosine tRNA. Eight loci can mutate to these suppressors. Therefore when one of them mutates, the product of the mutated gene should be a minor component of the total tRNA^{Tyr}. This can be investigated by sequencing tRNA^{Tyr} associated with strains possessing these suppressors, provided this species can be isolated to a high degree of purity. We have found that the two-dimensional electrophoretic separation of tRNAs on polyacrylamide gel³⁷ can be used routinely to isolate small amounts of extremely pure ³²P-tRNA^{Tyr} from yeast cultures (Fig. 1). The relative positioning of different tRNAs on the gels was sufficiently reproducible for the tRNA^{Tyr} spot (arrowed in Fig. 1) to be identified from the spot pattern, once it had initially been characterised on the basis of its fingerprint. The sequence analyses described here were carried out on the purified total tRNA^{Tyr} of the strains listed in Table 1.

S. cerevisiae strain L-133, with the highly efficient tyrosine-inserting amber suppressor *SUP5-a*^{24,32}, was examined for the presence of an altered tRNA^{Tyr}. As a control, tRNA^{Tyr} was also

Fig. 1 Purification of tyrosine tRNA from yeast. Cultures (50 ml) were grown at 30°C in nutrient low phosphate medium³⁴ containing 6 mCi of carrier-free ³²P-orthophosphate. Immediately before collection of each culture, a few cells were plated so that the proportion retaining the original suppressor at the time of collection could be determined subsequently by measuring suppression of auxotrophic and *iso-1*-cytochrome *c* nonsense mutants. Levels of *iso-1*-cytochrome *c* were used to distinguish between amber suppressors with different efficiencies of suppression. This was necessary because of the instability of suppressors and their tendency to mutate to less efficient forms³². Only cultures in which more than 90% of the cells retained the phenotype of the original suppressor were retained for the analyses. After the rest of the cells had been collected by centrifugation, RNA was extracted from the cell pellets³⁴, precipitated with ethanol and lyophilised. RNA samples were then dissolved in small volumes of 0.35 M NaCl, 0.01 M MgCl₂, 0.01 M sodium acetate, pH 5.0, and applied to 1 cm $\times$ 8 cm columns of benzoylated DEAE-cellulose previously equilibrated with the same buffer. tRNA was eluted in two consecutive steps, by applying to the columns first: 0.85 M NaCl, 0.01 M MgCl₂, 0.01 M sodium acetate, pH 5.0; and later: 1.0 M NaCl, 0.01 M MgCl₂, 20% ethanol, 0.01 M sodium acetate, pH 5.0. The tRNA fraction eluted by the ethanol-containing buffer was concentrated by ethanol precipitation, lyophilised and redissolved in 0.035 ml of gel sample buffer (60% sucrose, 4 M urea, 0.1 M sodium acetate, pH 5.0, 1% xylene cyanol FF). It was then separated by two-dimensional gel electrophoresis³⁵. tRNA spots were located by autoradiography of the wet gels, extracted³⁴ and individual tRNA species were identified from their fingerprints. *a*, Autoradiogram of a two-dimensional gel. The scales indicate the distances of migration (cm) in the first dimension (1) 10% gel and second dimension (2) 20% gel. The tRNA^{Tyr} spot is indicated by an arrow. *b*, Fingerprint of a ribonuclease T₁ digest of tRNA^{Tyr}. The RNA sample was purified on a two-dimensional gel, and individual oligonucleotides on the fingerprint are identified by their sequences. The first (1) and second (2) dimensions of electrophoretic separation³⁸ are indicated.

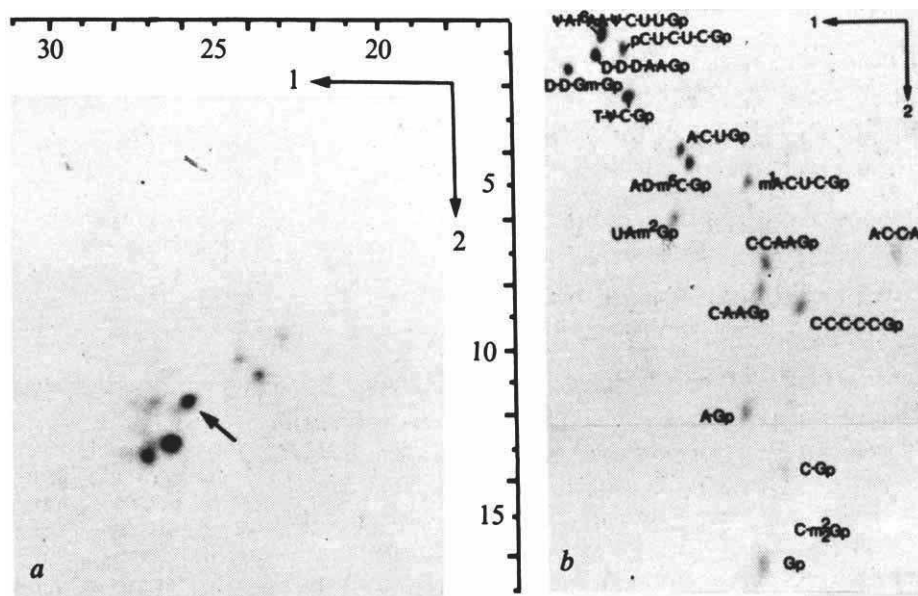


Table 1 Yeast strains

Strain no.	Abbreviated genotype	Derived from:	Suppresses	Suppressor inserts	Efficiency	Anticodon of tyrosine tRNA product of <i>SUP5</i> locus
SL210-3A	<i>sup</i> ⁺	—	None	—	—	GΨA
L-133	<i>SUP5-a</i>	<i>sup</i> ⁺	UAG	Tyrosine	high	GΨA→CΨA
SL171-2C	<i>SUP5-o</i>	<i>sup</i> ⁺	UAA	Tyrosine	high	GΨA→?
L-58	<i>SUP5-o→a</i>	<i>SUP5-o</i>	UAG	Not determined	high	?→CΨA
L-133a	<i>SUP5-a→a'</i>	<i>SUP5-a</i>	UAG	Not determined	low	Unknown

examined for the non-suppressing *sup*⁺ strain (SL210-3A) from which the *SUP5-a* mutant had been derived. No difference between the total tRNA^{Tyr} of these two strains could be discerned when T₁ or pancreatic ribonuclease digests were fingerprinted in two dimensions, using electrophoresis on cellulose acetate at pH 3.5 in the first dimension and on DEAE paper in 7% formic acid³⁶ in the second dimension, as in Fig 1b. If, however, T₁ ribonuclease digests were separated by homochromatography³⁶ in the second dimension, so as to effect better separation of the large oligonucleotides, a distinct difference in the fingerprints was apparent. Figure 2a shows a fingerprint of *sup*⁺ tRNA^{Tyr} digested with T₁ ribonuclease, and Fig. 2b and c shows the corresponding fingerprints of total rRNA^{Tyr} from two different *SUP5-a* cultures. A large oligonucleotide (marked C in Fig. 2b and c) is present in the T₁ ribonuclease digest of the *SUP5-a* tRNA^{Tyr} but is absent from the corresponding digest of the *sup*⁺ tRNA^{Tyr}. Since this fragment is not one of the normal digestion products of yeast tRNA^{Tyr}, its nucleotide sequence was determined. It was degraded by pancreatic ribonuclease (Fig. 3) to give Ai⁶AAΨp, ACp, Gp, Cp, Ψp, and Up. Also, after reacting the U, G and Ψ residues with carbodiimide reagent and analysing the products of subsequent pancreatic ribonuclease digestion³⁶, it was possible to show that this fragment contained the sequences (C)UC and (C)UUG (results not shown). Partial digestion with ribonuclease U₂ gave a series of fragments shown separated in Fig. 3, which were identified from their base compositions. The data obtained were only consistent with the nucleotide sequence: ACUCΨAi⁶AAΨCUUGp. Figure 4 shows that this oligonucleotide is derived from a tRNA^{Tyr} with anticodon CΨA. The species with such an anticodon would be expected to function as a UAG suppressor. Minor nucleotide analyses carried out in conjunction with the sequence analysis of fragment C showed that the second anticodon base of the suppressor tyrosine tRNA is pseudouridine, as in the wild-type

tRNA^{Tyr}. The weak intensity of the mutant anticodon spot C (Fig. 2b and c) indicates that only 5–8% of the total tRNA^{Tyr} from the *SUP5-a* strain existed in the form of this amber suppressor species, a level that is consistent with this species being the product of one of the eight genes for tyrosine tRNA. A UAG-specific suppressor can therefore be generated in both *E. coli*¹³ and yeast by mutation of the first base of the tyrosine tRNA anticodon.

The tRNA^{Tyr} from other amber suppressor alleles of *SUP5* was also examined. The *SUP5-o→a* allele was obtained²⁴ by mutation of the *SUP5-o* UAA suppressor, in contrast to the *SUP5-a* allele which was obtained directly from the *sup*⁺ wild type. But *SUP5-o→a* and *SUP5-a* are phenotypically indistinguishable and the sequence analyses of their tRNA^{Tyr} (Figs 2e and 3c and f) indicate that they are identical.

Since we have shown that these suppressors arise by mutation of the tyrosine tRNA anticodon, it is possible to study the structure and function of this species, as has been done with *E. coli* and bacteriophage tRNA suppressors. It has been shown that second base changes in the yeast amber suppressor tRNA^{Tyr} result in the suppressor functioning at a lower efficiency³². We have analysed one of these suppressors, *SUP5-a→a'* in strain L-133a which arose by an intragenic mutation of *SUP5-a* in L-133. In the fingerprint of tRNA^{Tyr} from *SUP5-a→a'* (Fig. 2f), the anticodon fragment of the amber suppressor tRNA^{Tyr} was either absent or present at an undetectable level. It is possible that the second site mutation of the *SUP5-a→a'* locus causes a reduced synthesis of suppressor tRNA, in much the same way as certain mutations of the *su*₃⁺ tRNA^{Tyr} gene of *E. coli* impair tRNA maturation³⁷.

Lack of identification of UAA-specific suppressor

Unlike bacterial ochre suppressors which recognise both UAA and UAG codons, yeast UAA suppressors act only on UAA

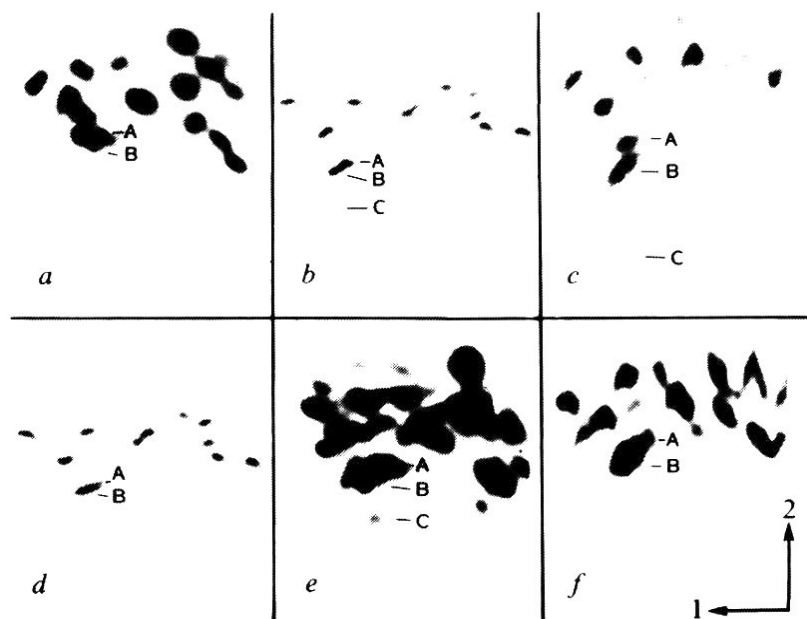


Fig. 2 T₁ ribonuclease digests of tyrosine tRNA isolated from: a, *SUP*⁺ (SL210-3A); b and c, two different cultures of *SUP5-a* (L-133); d, *SUP5-o* (SL171-2C); e, *SUP5-o→a* (L-58), and f, *SUP5-a→a'* (L-133a). Oligonucleotide separation was by electrophoresis on cellulose acetate in the first dimension (1) and ascending chromatography on DEAE-cellulose thin layers in the second dimension (2) using a 3% dialysed homomixture³⁸. A and B identify respectively the oligonucleotides pCUCUGp and ΨAi⁶AAΨCUUGp. C, ACUCΨAi⁶AAΨCUUGp, is the anticodon fragment of the amber suppressor tRNA^{Tyr}, present only in fingerprints (b), (c) and (e). Other differences between the fingerprints are due to differences in the resolving power of homomixtures and fluctuations in the extent to which T₁ ribonuclease has cleaved the fragment Cm₂GCAAGp into Cm₂G>p and CAAGp.

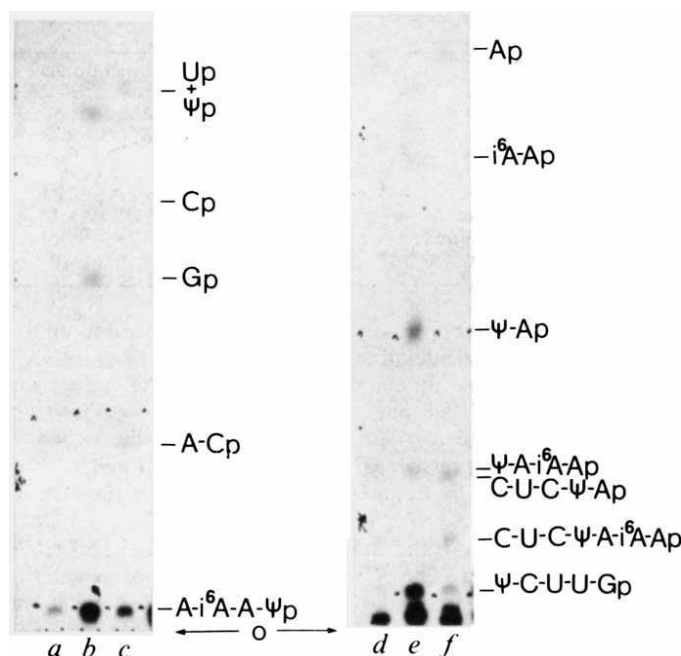


Fig. 3 Pancreatic and U_2 ribonuclease digests of the anticodon oligonucleotides of the UAG suppressor $tRNA^{Tyr}$. *a* and *c*, Pancreatic ribonuclease digests of $ACUC\Psi Ai^6 AA\Psi CUUGp$, the anticodon loop fragments of the amber suppressor $tRNA^{Tyr}$, derived, respectively, from strains $SUP5-a$ and $SUP5-o \rightarrow a$. *b*, Marker digest of $\Psi Ai^6 AA\Psi CUUGp$. Separation was by electrophoresis, 1 kV for 3 h, on DEAE paper at pH 3.5. *d* and *f*, U_2 ribonuclease digests of the anticodon loop fragment of amber suppressor $tRNA^{Tyr}$ derived from $SUP5-a$ and $SUP5-o \rightarrow a$, respectively. *e*, Marker U_2 digest, of $\Psi Ai^6 AA\Psi CUUGp$. Digestion was with 0.05 ml of U_2 ribonuclease ($0.1 U ml^{-1}$), 0.05 M sodium acetate, pH 4.5, for 4 h at $37^\circ C$, and separation was by electrophoresis, 1 kV for 6 h on DEAE paper in 7% formic acid.

and not on UAG codons. The only *E. coli* ochre suppressor which has been sequenced is the $tRNA^{AocTyr}$. This species has the anticodon $U^*UA^{14,38}$ and should therefore decode both UAA and UAC to UAG according to the 'wobble' theory of codon-anticodon recognition³⁹. A mutant suppressor glutamine tRNA of phage T_4 , with NUA anticodon, also recognises both UAA and UAG^{15,40}. There have been two proposals to explain how an ochre suppressor $tRNA^{Tyr}$ of yeast might be able to decode UAA but not UAG. According to the first hypothesis⁴¹, the anticodon is presumed to be $I\psi A$ (topaz) obtained by mutation of the anticodon to AUA, followed by enzymatic modification, thus giving a tRNA which should translate UAA in addition to the two normal tyrosine codons. The second hypothesis²¹ is that the mutant $tRNA^{Tyr}$ has the anticodon $S\psi A$ (sepia) where S is 2-thio-5-carboxymethyluridine methyl ester. This modified nucleoside has been identified as the first anticodon base in $tRNA_{3}^{Glu}$ (ref. 42) and $tRNA_{2}^{Leu}$ (refs 43, 44) from yeast. In this position it pairs preferentially with adenosine rather than guanosine in the third position of a codon. A third possibility might be that a weak recognition of UAA by $tRNA^{Tyr}$ is enhanced by a base change outside the anticodon. This is known to be the case for UGA recognition by a suppressor $tRNA^{Trp}$ of *E. coli*¹⁷. An anticodon change, however, is much more probable in view of the demonstration that tyrosine-inserting ochre suppressors of yeast can mutate to allelic amber suppressors.

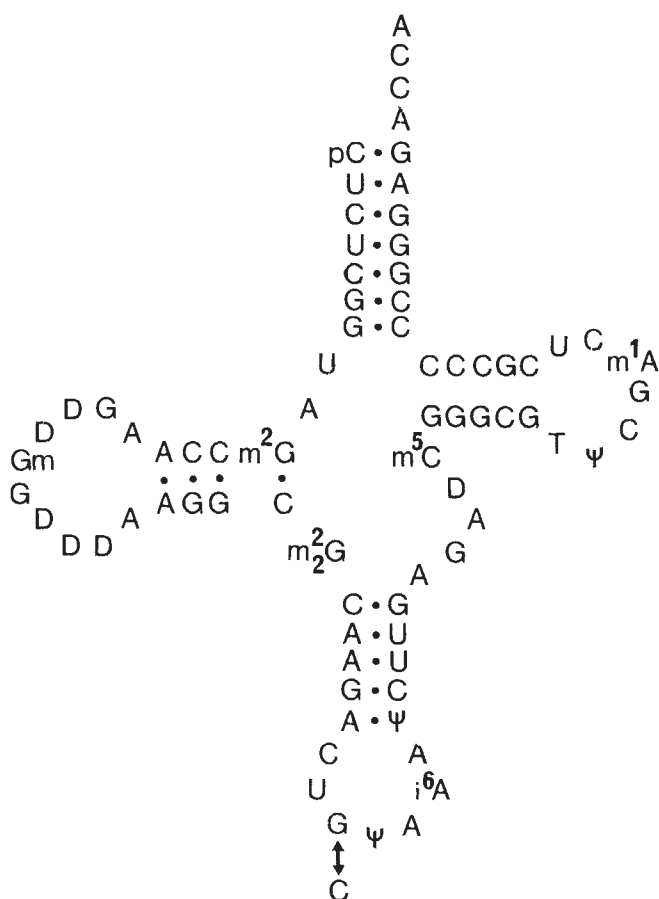
To explore these possibilities, we fingerprinted $tRNA^{Tyr}$ from strain SL171-2C which carries a tyrosine-inserting ochre suppressor, $SUP5-o$, that is allelic to the amber suppressor $SUP5-a$. According to the second hypothesis, the UAA suppressing $tRNA^{Tyr}$ should yield the fragment $ACUS\Psi Ai^6 AA\Psi CUUGp$ on digestion with T_1 ribonuclease. This oligonucleotide differs in only one base from the anticodon fragment of the $SUP5-a$

$tRNA^{Tyr}$. Assuming the unstable S nucleoside does not produce anomalous behaviour, such an oligonucleotide should occupy almost the same position on the fingerprints as the amber suppressor anticodon at spot C. In the fingerprint of the $SUP5-o$ $tRNA^{Tyr}$ (Fig. 2*d*) no spot could be seen in this position. If, however, the ochre suppressor has an $I\psi A$ anticodon, then the sole difference between a T_1 ribonuclease digest of this species and that of normal $tRNA^{Tyr}$ will be the presence of $ACU\psi p$ in place of $ACUGp$ (Fig. 1*b*), since the enzyme cleaves polynucleotides after both G and I. Because of technical difficulties in identifying a small amount of a ^{32}P -labelled minor nucleotide from a very small fraction of the $SUP5-o$ $tRNA^{Tyr}$, we did not pursue this analysis further.

Interpretation

Nucleotide sequencing established that the yeast amber suppressor $SUP5-a$ determines a mutant form of $tRNA^{Tyr}$ which has the anticodon $C\psi A$ instead of the normal $G\psi A$. This mutationally altered anticodon is consistent with amber suppressibility according to the wobble hypothesis and it corresponds to the anticodon of a $tRNA^{Tyr}$ amber suppressor of *E. coli*. Although we were unable to detect the nucleotide changes of $tRNA^{Tyr}$ associated with the UAA suppressor $SUP5-o$, the amber suppressor anticodon was clearly absent. The nature of the anticodon in the $SUP5-a$ $tRNA^{Tyr}$ could be inferred from the observation that the $tRNA^{Tyr}$ corresponding to the amber suppressor $SUP5-o \rightarrow a$, which was derived by a single-step mutation from $SUP5-o$, seemed to have an identical sequence to $SUP5-o$ $tRNA^{Tyr}$ which was derived by a single-step mutation from the wild-type gene. Assuming that the mutations occurred by single-base changes, the simplest interpretation of these results is that the UAA suppressor is a

Fig. 4 Primary structure of yeast $tRNA^{Tyr}$ (ref. 35) showing the anticodon base substitution present in the amber suppressor $tRNA^{Tyr}$ species.



tRNA^{Tyr} having an alteration of the first base in the anticodon, although the base in question has not been identified.

This work was supported in part by grants from Landforeningen til Kraeftens Bekaempelse, the Danish Research Council, NIH the American Cancer Society and ERDA. P.W.P. was supported by an EMBO fellowship and M.W. by a Danish Government scholarship.

Received May 31; accepted June 29, 1976.

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Systematic approach to the correction of intonation in wind instruments

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The effect of perturbations in the bore diameter on the intonation of wind instruments is investigated. From measurements of the bore and the amplitude of the standing waves, a modified bore shape is calculated to give any desired correction in intonation.

MUSICAL instruments have evolved over the centuries very much by trial and error. In spite of scientific investigation, many undetermined factors critically affect the performance of instruments, and empirical methods of design are still necessary. Some features, however, are easily amenable to quantitative treatment, and we present here the results of some calculations and experiments on improving the intonation and tone colour of the trumpet.

All brass instruments have acoustical and mechanical inadequacies for which most players automatically compensate¹. To the average listener an instrument may sound perfectly in tune but this largely results from the competence of the player who may be able to 'lip' a note by ± 1 semitone, usually at the expense of tone colour. Similarly, the intonation of inexperienced players tends to be more affected by the deficiencies of their instruments.

The frequency of a note produced by a trumpet is determined by a complex interaction between the vibrating air column in the instrument and the player's lips. It is also affected by acoustical feedback to the player's ears, and differences of more than 10 cents (1 cent = 0.01 tempered semitone) occur when different players use the same instrument. The frequency

is, however, always very close to a resonance frequency of the air column in the trumpet.

The necessity of first eliminating the contribution of the individual player to the intonation has been recognised by several workers^{2–7}, but no satisfactory method of artificial excitation has yet been described. We have used a modification of the method originally described by Webster³ to measure the resonance frequencies of the air column in the trumpet. The apparatus (Fig. 1) operates over the frequency range C₂ (65 Hz) to B₇ (3,951 Hz) (C₄ = middle C) and includes an automatic locking device for the location and tracking of resonance peaks. It gives a digital display and printed record of the amplitude and intonation of each resonance (in cents) relative to the equitempered scale.

It was found that the frequency of each resonance of an instrument corresponded closely with the mean of the frequencies produced for this note by a number of players, so that observations with this apparatus can therefore be used for the basis of quantitative measurement of the intonation of the instrument.

Resonances and tone colour

The resonance frequencies are principally determined by the shape of the bore of the trumpet, and evolution has produced a bore shape with resonances fairly close to the appropriate notes of the equitempered scale. Unfortunately intonation is not the only consideration in fixing the resonances as the following example shows.

Figure 2 shows the first 10 resonances of the tube with their corresponding equitempered frequencies. When the trumpet is

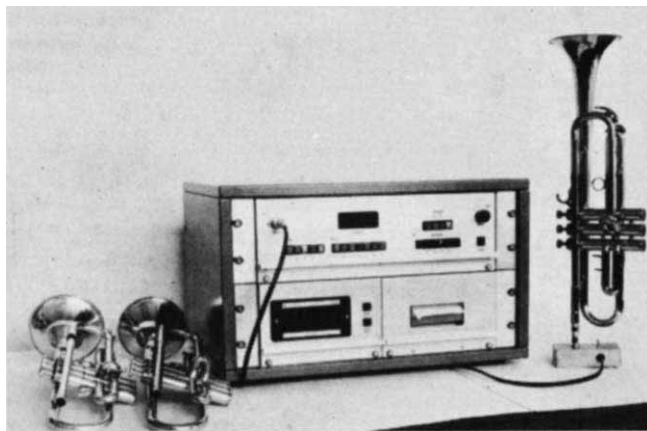


Fig. 1 The apparatus designed to locate automatically and track the resonances of a wind instrument. Digital displays indicate the intonation and amplitude of the resonances.

sounding a steady note, D_5 (587.33 Hz), the frequency of the second harmonic of this fundamental is 1,174.7 Hz, and this strongly excites the resonance at 1,174.7 Hz. This resonance is also near the fourth harmonic (at 1,165.4 Hz) of the fundamental Bb_3 (233.08 Hz). But because this harmonic misses the resonance peak by 14 cents, its strength is not so strongly reinforced, and this has a significant effect on the tone colour of Bb_3 .

In practice, the harmonics of any note fall near but not exactly on the resonances of the tube, and are, to varying degrees, reinforced by resonance. The resonances strongly affect the tone colour of notes, and hence the choice of a set of resonances is inevitably a compromise between their intonation when used as fundamentals, and their reinforcement of the harmonics of other notes. We are not concerned here with this compromise, but we show how the design of the bore shape can be modified to move selected resonance frequencies by small amounts to desired values.

Perturbations in the bore

It has been known for many years that small changes in the bore cross section near a node or antinode of the standing wave will change the resonance frequency. A decrease in area at a pressure antinode produces an increase in that frequency, and an increase in area gives a frequency decrease. At a pressure node the effects of changing the area are reversed. We have used perturbation theory to calculate the change in a given bore shape necessary to produce prescribed changes in each of the resonance frequencies.

The pressure oscillations $P_n(x)$, at frequency f_n in a standing wave in the air column in a horn, of cross section $S(x)$, at a

distance x from one end, are described by the approximate wave equation⁸

$$\frac{d}{dx} \left[S(x) \frac{dP_n}{dx} \right] + \frac{4\pi^2 f_n^2}{c^2} S(x) P_n(x) = 0 \quad (1)$$

where c is the velocity of sound. The boundary conditions at the mouthpiece and open end are assumed to be

$$P(x) = \beta \frac{dP}{dx} \bigg|_{x=0,L}$$

where the constant β may be different at the two ends of the instrument but the values are assumed to be independent of the mode of oscillation. This is a standard Sturm-Liouville equation and the solutions form a complete orthonormal set⁹.

If the cross section $S(x)$ is perturbed by a small change $\delta S(x)$ and $s(x) = \delta S(x)/S(x)$ the first-order change in the resonance frequencies can be shown to be

$$\frac{\delta f_n}{f_n} = \int_0^L s(x) G_n(x) dx \quad (2)$$

where

$$G_n(x) = \frac{1}{2} S(x) \left\{ \frac{c^2}{4\pi^2 f_n^2} \left[\frac{dP_n}{dx} \right]^2 - P_n^2(x) \right\} \quad (3)$$

In deriving this, it is necessary to take δS zero at both ends of the instrument so that the boundary conditions are not changed. The values of $P_n(x)$ used above are taken to be normalised so that

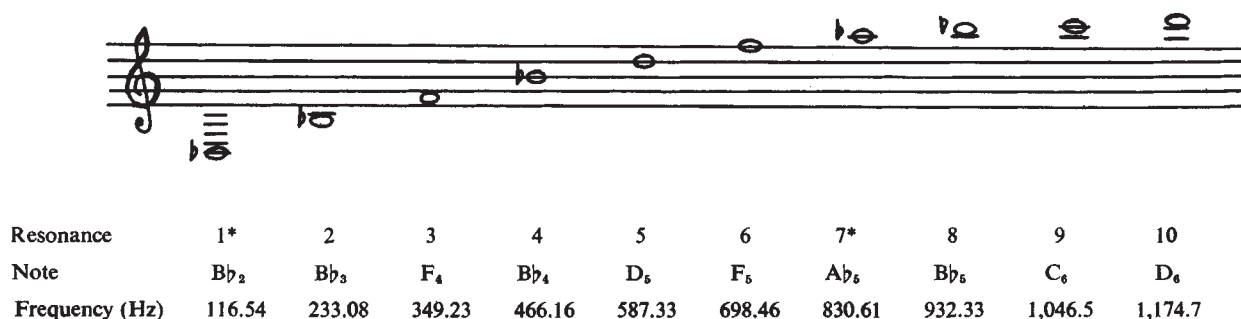
$$\int_0^L S(x) P_n^2(x) dx = 1 \quad (4)$$

This implies that the dimensions of P are $L^{-3/2}$, and not those of pressure. An alternative expression for $G_n(x)$ can be derived using equation (1)

$$G_n(x) = \frac{c^2}{16\pi^2 f_n^2} \frac{d}{dx} \left[S(x) \frac{d}{dx} P_n^2(x) \right] \quad (5)$$

We wish to prescribe δf_n and calculate $\delta S(x)$. It is clear that the solution is not unique and we shall seek the 'smoothest' change in the bore that produces the required frequency shifts. It may be inconvenient to make changes over the whole instrument, because of the valves and tuning slides, and we shall restrict them to the section $x = 0$ to $x = l$. To avoid discontinuities in the bore we need $\delta S(0) = 0$ and $\delta S(l) = 0$.

Fig. 2 The first 10 resonances of a trumpet with their associated equitempered frequencies. *Not normally used.



A suitable measure of smoothness is the mean square derivative

$$\int_0^l S(x)[s'(x)]^2 dx$$

and we minimise this, subject to the constraints that $s(x)$ produces the required frequency shifts using Lagrange's method of undetermined multipliers, that is we minimise

$$\int_0^l S(x)[s'(x)]^2 dx - \sum_{m=1}^N \lambda_m \left[\frac{\delta f_m}{f_m} - \int_0^l s(x) G_m(x) dx \right]$$

The Euler equation for this problem is

$$\begin{aligned} \frac{d}{dx}[S(x)2s'(x)] &= \sum_{m=1}^N \lambda_m G_m(x) \\ &= \frac{c^2}{16\pi^2 f_m^2} \sum_{m=1}^N \lambda_m \frac{d}{dx} \left[S(x) \frac{d}{dx} P_m^2(x) \right] \end{aligned}$$

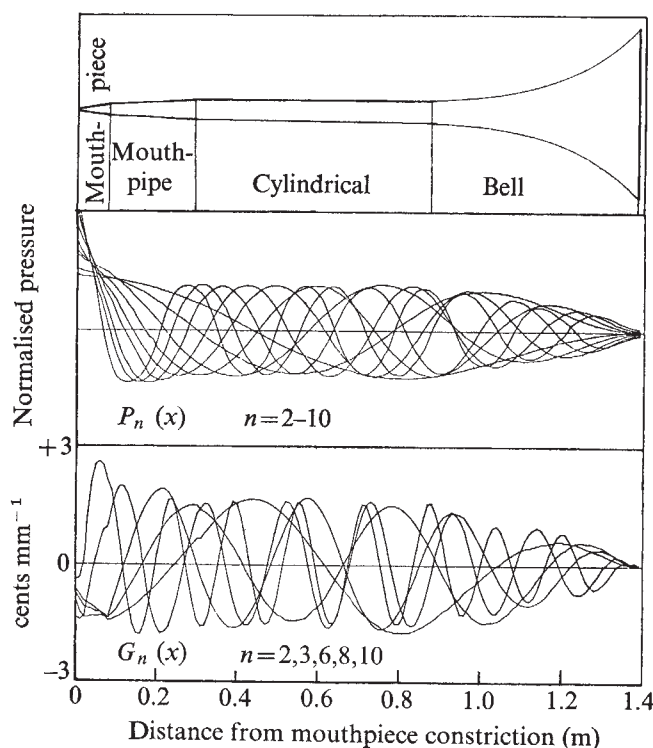
Integrating twice gives

$$s(x) = \sum_{m=1}^N \mu_m P_m^2(x) + A \int_0^x \frac{d\xi}{S(\xi)} + B \quad (6)$$

A and B are arbitrary constants of integration and they can be chosen to make $\delta S(0) = 0$ and $\delta S(l) = 0$, and μ_m is a multiple of λ_m .

Substituting this into (2) gives N linear equations which, together with the conditions on δS , determine the $N+2$ constants μ_m , A , B . Equation (6) then gives the required change in bore.

Fig. 3 The physical shape of a trumpet (not to scale) with $P_n(x)$ and $G_n(x)$ for selected modes.



The reader may wonder why we have used the 'smoothest' perturbation rather than the 'smallest' one. If we define

$$\varepsilon = \int_0^l [s'(x)]^2 dx$$

and find the function $s(x)$ that minimises this and produces the required frequency shifts we get

$$s(x) = \sum_m \mu_m G_m(x) \quad (7)$$

This cannot, however, be used as a solution to our problem as it does not ensure that $\delta S(0) = 0$ and $\delta S(l) = 0$.

The eigenfunctions for the standing waves

To perform the calculations of the previous section the functions $P_n(x)$ are required. Although it is possible, in principle, to calculate these given the cross-sectional area of the trumpet, it is undoubtedly easier to observe them.

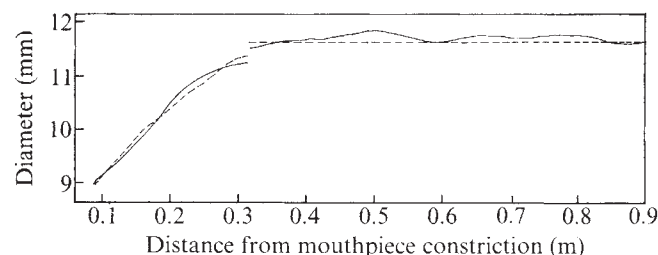


Fig. 4 The bore shape of a trumpet (conical and cylindrical portions) before and after perturbation. —, Perturbed bore; ---, original bore.

In a standing wave, when damping is very small, the oscillations have the same phase at all points between adjacent nodes, and the phase changes by π across each node. The result is not exact if damping is included, but as we have neglected damping, even in equation (1), there is no advantage in observing the phase of the pressure oscillations, and we assume the phase is 0 or π . The function $P_n(x)$ is therefore given by the observed pressure amplitude between alternate pairs of nodes and minus the observed pressure in the gaps.

The derivative dP_n/dx is also required. Because of the well known dangers of numerical differentiation we obtain this by integrating equation (1) giving

$$\frac{dP_n(x)}{dx} = -\frac{4\pi^2 f_n^2}{c^2 S(x)} \int_0^x S(\xi) P_n(\xi) d\xi + \frac{C}{S(x)}$$

The value of the constant of integration C is found by integrating again, thus introducing another constant, and finding both constants by satisfying the equations at two particular values of x .

Varying the length of pipe over which the perturbation is made

An interesting result can be derived about the mean square perturbation, which can be evaluated using equation (7). This can be differentiated with respect to l , bearing in mind that the numbers μ depend on l . The derivatives of μ can be eliminated by differentiating equation (2) and we get the remarkable result

$$\frac{\partial \varepsilon}{\partial l} = -[s(l)]^2 \quad (8)$$

It follows that $\partial \varepsilon / \partial l$ is never positive, and the mean square value of the perturbation required always decreases as we

spread the perturbation over longer lengths of the tube.

Asymptotic results

The high eigenvalues of a Sturm-Liouville equation have simple asymptotic distributions⁹. When n is large, $f_n \sim nc/2L$ and

$$P_n(x) \sim \left(\frac{2}{LS(x)}\right)^{1/2} \cos\left[\left(n-\frac{1}{2}\right)\frac{\pi x}{L} + \theta_n\right]$$

in which θ_n depends on the boundary conditions. A musical instrument like the trumpet has evolved into a shape such that the frequencies of the resonances are almost uniformly spaced. In other words, the formula $f_n \sim nc/2L$, which can be proved for large n , is in fact almost exact for all n . This suggests that the associated asymptotic eigenfunctions, $P_n(x)$, might be good approximations for all n . This would avoid the difficult measurement of $P_n(x)$. If the boundary conditions in the trumpet are approximately

$$P_n(L) = 0 \text{ and } \left[\frac{dP_n}{dx}\right]_{x=0} = 0$$

we can show that

$$G_n(x) \sim -\frac{1}{L} \cos\left(\frac{(2n-1)\pi x}{L}\right)$$

The integrals involved in the calculations can now be evaluated analytically.

We have tested the usefulness of this by prescribing some values for δf_n , calculating the bore shape required using the asymptotic expression for $G_n(x)$ and then calculating the exact frequency shifts (equation 2) that this bore shape would produce, using the correct observed value of $G_n(x)$. The changes produced are found to be in the right direction, but the magnitude of error is large. The asymptotic results can, however, be usefully employed if (a) a rapid approximate answer is required: the measurement of $P_n(x)$ is time consuming, or (b) if a very high harmonic needs adjustment, where the observations of the resonance are impossible.

Verification of the theory

The trumpet was driven by the automatic tuner described in the first section and the sound pressure was measured in two ways; using probe tubes inserted radially through holes in the bore at each centimetre of the instrument's length and using a long flexible probe drawn through the instrument. There was little variation between the results of either method and they are shown with the computed functions $G_n(x)$ in Fig. 3.

An earlier section has indicated the difficulties involved in deciding upon the optimum intonation of an instrument. To verify our ideas, however, these arbitrary changes were chosen:

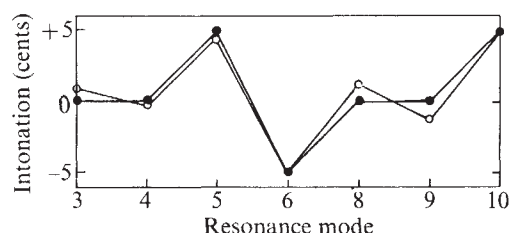


Fig. 5 Relative intonation of seven resonance modes. ●, Required change in intonation; ○, change obtained by perturbation.

for the fifth and tenth order resonance, a change of +5 cents for the sixth order resonance a change of -5 cents, and all other resonances to be unaltered. Similarly, the perturbed length was arbitrarily restricted to the first 0.9 m of the instrument. This bore provides the 'open' notes of the instrument and additional tubing is inserted by valves to produce a chromatic scale. Consequently the effect of our perturbation is likely to be less accurate as more tubing is added; the perturbation method could be applied to this valve tubing for further

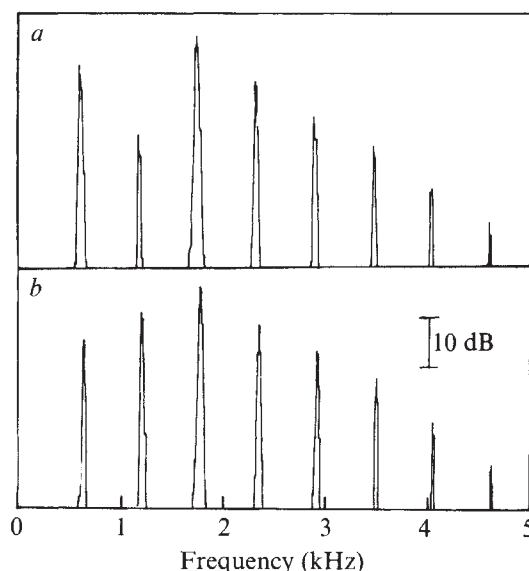


Fig. 6 The spectra of note D₅ (587.33 Hz) blown by a professional musician. (a) before, and (b) after perturbation. Both notes were played *mezzo forte* in an anechoic chamber with the microphone at 1 m along the axis of the instrument.

correction to notes using valve tubes. Equation (8) implies that because of the relatively short length of the valve tubing, only small intonation corrections could be made in this way without unacceptably large diameter changes.

A graph of the new bore shape (Fig. 4) indicates a maximum change in diameter of < 0.2 mm. This may be compared with a maximum of 0.14 mm for the smallest mean square perturbation mentioned earlier.

The new shape was reproduced with glass reinforced plastic and its resonances measured using the automatic tuner. There is close correspondence between the required and measured intonation (Fig. 5); the small errors mainly arise from the difficulty in producing a new bore to an accuracy of better than 0.02 mm.

It can be appreciated from the qualitative effects of diameter changes at nodes and antinodes that some prescribed frequency shifts may require conflicting diameter changes. The result in these cases is that very large increases and decreases in diameter are required over short distances. These would be unacceptable for a musical instrument and would also render our first-order perturbation theory invalid. Our optimisation procedure makes sure that large changes in diameter are not used unnecessarily.

For comparison another calculation has been made which assumes a non-optimum bore shape given by a superposition of sine waves. The maximum diameter change required here was 3 mm.

Curing a faulty trumpet

Although the foregoing theory is not ideally suited to the complete acoustical design of a new instrument, the best application of this work is with the improvement of the

individual notes of prototype or production instruments. One such instrument was found to have a weak second harmonic when playing D_5 (587.33 Hz) (Fig. 6a). As already mentioned, this harmonic uses the tenth resonance for reinforcement; and this was found to be flat with respect to the fifth. The tenth resonance is not normally played as a fundamental and hence can be altered to improve the tone colour. Using our new technique we were able to raise this tenth resonance by ten cents to give the improved response (Fig. 6b).

This work was supported in part by the SRC and Boosey & Hawkes Ltd. We thank Dr D. M. A. Mercer for encourage-

ment and also acknowledge the assistance of Mr W. Tompkins and the trumpet player, Michael Laird.

Received May 13; accepted July 21, 1976.

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letters to nature

Does Mercury have a molten core?

THE discovery of an intrinsic magnetic field for the planet Mercury by Mariner 10 (ref. 1) and its preferred source in an internal dynamo² imply the existence of a conducting molten core. We propose here a feasible, non-seismic observational experiment to determine the existence and extent of such a molten core, which would be vital for calculations of Mercurian thermal histories and dynamo field generation.

Siegfried and Solomon³ have calculated two thermal histories for Mercury leading to a differentiated and an undifferentiated planet respectively. In the latter models, of course, Mercury would have no core. Even in the differentiated model a once molten core tends to solidify as the planet cools. Whether or not a given differentiated model leads to a molten core persisting to the present depends critically on assumptions about initial conditions (the time of core formation), the time varying distribution of radioactive elements within the planet, thermal conductivities and the extent and efficiency of convective heat transport. Even with all the radioactive elements being removed from an initially differentiated molten core, Fricker *et al.*⁴ have shown that a liquid outer core 500-km thick may still persist if one accounts for a thermal barrier at a liquid iron-solid silicate core-mantle boundary. The thermal barrier results from the higher melting point and lower thermal conductivity of the silicates—the liquid iron core temperature rises above the melting curve near the core-mantle boundary thereby decreasing the core temperature gradient. If the solid mantle is allowed to convect, however, it must contain a density of heat sources comparable to the Earth's mantle-wide average if the core is to remain molten⁵.

Since we cannot be certain about the convective heat transport or the distribution of heat sources, theoretical predictions about the nature of the Mercurian core are not conclusive. There is thus a severe need for the experimental determination of some of the key internal parameters or, more directly, for an experiment which would determine unambiguously the extent of any fluid core. The latter would motivate considerable research into the generation of planetary magnetic fields and would place bounds on the internal parameters related to the thermal history—whether or not the core was found to be molten. Fortunately, the effects of a liquid core on the dynamics of Mercury's rotation affords a technically feasible, albeit expensive means of determining unambiguously the nature of Mercury's core⁶.

There are two necessary conditions for this scheme to work. First, a liquid core must not follow the mantle's forced librations (88-d period) in longitude, which arise from the periodic solar torque on its asymmetric mass. Second, the core must follow the mantle on the time scale of the 250,000-yr precession. We shall assume these two conditions are satisfied to develop the

method and subsequently establish bounds on the core viscosity for which they are satisfied.

The amplitude of the physical librations in longitude with the period of a Mercury year is⁷

$$\phi_0 = \frac{3}{2} \left(\frac{B-A}{C_m} \right) \left(1 - 11e^2 + \frac{959}{48}e^4 + \dots \right) \approx \frac{B-A}{C_m} \quad (1)$$

where $A < B < C$ are principal moments of inertia of the entire planet, e is the orbital eccentricity and C_m is the moment of inertia of the mantle alone. The physical libration is thus larger than it would have been if the core were solid. (The core is assumed axially symmetric, so it does not contribute to $B-A$.) The tides carry Mercury to a configuration which is a generalisation of Cassini's laws for the Moon⁸, where the spin axis, orbit normal and normal to the invariable plane remain coplanar as the first two vectors precess around the last with a 250,000-yr period. In this 'Cassini state', the following relation is satisfied^{8,9}

$$K_1(\theta) \left(\frac{C-A}{C} \right) + K_2(\theta) \left(\frac{B-A}{C} \right) = K_3(\theta) \quad (2)$$

where K_1, K_2, K_3 are constants involving the orbital eccentricity, inclination and mean motion and the planetary spin angular velocity and explicitly the obliquity θ . In equation (2), $K_2 \ll K_1$ and can sometimes be neglected as it has been in the classical determination of $(C-A)/C$ for the Moon⁸. Note that C and not C_m is appropriate here, because of our assumption that any liquid core is able to keep up with the mantle precession. Also, θ and ϕ_0 are measurable quantities.

The second degree gravitational harmonics are related to the moment of inertia differences by

$$J_2 = \frac{C-A}{MR^2} - \frac{1}{2} \frac{(B-A)}{MR^2} \quad (3)$$

$$C_{22} = \frac{B-A}{4MR^2}$$

where M and R are the planetary mass and radius respectively. Equations (3) can be solved for $(B-A)/MR^2$ and $(C-A)/MR^2$ in terms of J_2 and C_{22} , where the latter two quantities can be determined by tracking one or more artificial satellites. Substitution of the solutions of equations (3) into equation (2) yields a numerical value for C/MR^2 since the K are known once the obliquity θ is determined. This number, already determined for the Moon by the same procedure¹⁰, is a measure

of the density distribution in the planet. ($C/MR^2 \approx 0.33$ for the Earth, ≈ 0.40 for the Moon, ≈ 0.40 for an homogeneous sphere.)

Finally, measurement of the amplitude of the physical libration ϕ_0 yields $(B-A)/C_m$ from which three known factors yield

$$\left(\frac{C_m}{B-A}\right)\left(\frac{B-A}{MR^2}\right)\left(\frac{MR^2}{C}\right) = \frac{C_m}{C} \leq 1 \quad (4)$$

A value for C_m/C of 1 would indicate the core to be firmly coupled to the mantle and hence most probably solid. If the entire core or the outer part is fluid, $C_m/C \approx 0.5$ for the large core size ($R_c \approx 0.75R$) in current models of the interior⁵.

How good are the assumptions that a fluid core will not follow the short period physical librations but will follow the long period precession? The appropriate time scale to compare with the 88-d and 250,000-yr periods is the 'spin up' or 'spin down' time for the core fluid (rotating at a slightly different angular velocity) to approach the perturbed angular velocity of the mantle¹¹.

$$\tau = R_c/(v\dot{\psi})^\dagger \quad (5)$$

where R_c is the core radius, v is the kinematic viscosity and $\dot{\psi}$ is the spin angular velocity of the planet. If $\tau > 88$ d the core will not follow the mantle libration, whereas if $\tau < 250,000$ yr, the core will follow the mantle precession. These bounds on τ lead to the bounds on v of

$$8 \times 10^{-4} < v < 2 \times 10^9 \text{ cm}^2 \text{ s}^{-1} \quad (6)$$

where both necessary conditions for the determination of C_m/C are satisfied if v is within these bounds. This is almost certainly true since the range in equation (6) includes the complete range estimated for the Earth's core of 5×10^{-3} – $10^7 \text{ cm}^2 \text{ s}^{-1}$ (ref. 12).

The numbers ϕ_0 , θ , J_2 and C_{22} must be determined to find C_m/C and hence the extent of the Mercurian liquid core. The first two are $O(20''\text{--}40'')$ and $O(\lesssim 1^\circ)$ (ref. 9, and B. A. Smith, personal communication) respectively and will almost certainly require rather sophisticated instrumentation on the surface of the planet. The latter two can be obtained from precise tracking of artificial satellites. The design of instrumentation to measure the small angles ϕ and θ which is within the weight limitations and which will survive the possibly hard landing of any spacecraft sent to Mercury's surface is a severe challenge. The large scientific return which could, however, be realised from such surface instrumentation means that the attempt at the design should be made.

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Received March 4; accepted July 7, 1976.

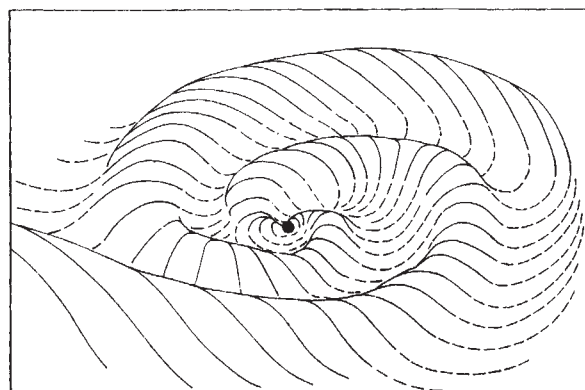
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Structure of the extended solar magnetic field and the sunspot cycle variation in cosmic ray intensity

THE interplanetary magnetic field within several astronomical units of the Sun appears to have one polarity in most of the hemisphere north of the solar equatorial plane and the opposite polarity in most of the hemisphere south of the equatorial plane^{1–7}. The two hemispheres are separated by a curved current sheet that typically crosses the solar equatorial plane in either two or four places, thus dividing the equatorial region into either two or four sectors. Near sunspot minimum, at 1 AU the curved current sheet has a spread in latitude of typically $\pm 15^\circ$, so that the sector boundary (the current sheet separating the two hemispheres of opposed field polarity) is almost parallel to the solar equatorial plane. In the photosphere, on the other hand, the sector boundary makes an angle of $\sim 90^\circ$ with the equatorial plane⁸. At $1.5R_\odot$, in 1972 and 1973, the angle between the sector boundary and the equatorial plane was $\sim 45^\circ$ (ref. 9), and at $3\text{--}10R_\odot$ the angle between boundary and plane was $\sim 25^\circ$ (ref. 10). A schematic diagram of this structure for the case of four sectors is shown in Fig. 1. We here propose that a connection exists between the extent of these magnetic fields and the observed variations in cosmic ray intensity at the Earth.

In the photosphere, near sunspot minimum, the sector magnetic fields cover a range in latitude of typically $\pm 40^\circ$ (ref. 8), while at 1 AU the comparable range in latitude has been compressed to perhaps $\pm 15^\circ$. How is this compression in latitude accomplished? A typical magnitude of the sector magnetic fields in the photosphere is 0.5 gauss (P. H. Scherrer and T. L. Duvall, personal communication). This is a measure of the large scale field that will dominate in the region a few $R_\odot$ above the photosphere, where the smaller scale but much stronger fields associated with active regions do not reach. In the polar regions of the Sun the large scale unidirectional photospheric field has a typical magnitude of perhaps 5 gauss (R. Howard, personal communication). Thus, at 1 or $2R_\odot$

Fig. 1 Schematic showing the warped current sheet in the inner Solar System (inside 6 AU). This current sheet divides the interplanetary magnetic field in the heliosphere into two regions with oppositely directed field lines. In one region the field polarity is away from the Sun (at present this region is north of the solar equator), in the other region the field polarity is toward the Sun. The situation is shown for a four-sector structure, that is, as the current sheet is rotated past a stationary observer in the course of a solar rotation, the observer will see four changes of magnetic polarity, suggesting that the interplanetary magnetic field is divided into four sectors of alternating polarity. Where the current sheet lies above the solar equatorial plane it is shown by full lines, while dashed lines indicate that the current sheet is below the equatorial plane. The extent in latitude of the current sheet was assumed to be $\pm 15^\circ$. The Sun at the centre is not shown to scale.



above the photosphere, and above the region of influence of active regions, the magnetic pressure associated with the solar polar regions is two orders of magnitude larger than the magnetic pressure associated with the equatorial sector structure. This will compress the equatorial sector field structure into a narrow range of latitudes. This effect can be clearly seen in the sketch in Fig. 2 made from an eclipse photograph taken at solar minimum in 1954 (ref. 11).

During an interval near sunspot maximum, when the solar polar field polarities are reversing, the magnitude of the polar fields may be considerably less, and the resulting compression of the equatorial field structure also much less. The sector structure fields may then occupy a much larger fraction of the heliosphere. Since the sector structure fields reverse polarity typically four times or two times per solar rotation, a galactic cosmic ray headed towards the Sun may encounter considerably more magnetic scattering from the complex sector structure field than from the unidirectional field that fills most of each solar hemisphere near sunspot minimum. This geometrical effect may be the principal cause of the 11-yr modulation of cosmic-ray intensity observed at the Earth, since the solar-wind velocity¹² and the magnitude of the interplanetary field¹³ observed near the Earth have not changed very much during the present sunspot cycle.

The fraction of the heliosphere occupied by sector-structure fields as a function of time through an average sunspot cycle can be estimated in the following way. Because the current sheet shown in Fig. 1 is warped with respect to the solar equatorial plane, during the half year when the Earth is north of the equatorial plane an interplanetary sector with the same polarity as the northern solar polar region is observed to be wider than it would be if observed when the Earth is in the equatorial plane of the Sun. For example, assume that there are two sectors per solar rotation and that the extent in heliographic latitude of the sector structure near the Earth is $\pm 20^\circ$. When the Earth is at a latitude near 7°N (near September 7), the sector whose polarity is the same as the northern polar region will be observed to last 16.5 d, as compared with the 13.5 d it would last if observed when the Earth is near the solar equatorial plane. For comparison, if the extent in heliographic latitude of the sector structure near the Earth is $\pm 45^\circ$, then the sector with the same polarity as the northern polar region will have a length of 14.6 d when observed near the Earth at 7°N . We see that if we measure the magnitude of this Rosenberg-Coleman effect^{14,15} through a sunspot cycle we can estimate the extent in heliographic latitude of the sector structure.

We have used an harmonic analysis to compute the average amplitude of the Rosenberg-Coleman effect as a function of years from the time of sunspot minimum for the four sunspot cycles whose minima were near 1934, 1944, 1954 and 1965. Interplanetary field polarities inferred from polar geomagnetic variations¹⁶ were used, and the resulting amplitude of the Rosenberg-Coleman effect was multiplied by 1.43 to correct for the $\sim 85\%$ accuracy^{17,18} of the inferred interplanetary field polarities.

Figure 3 shows the resulting value for the extent in heliographic latitude of the sector structure through an average sunspot cycle. Three-year running means were used to reduce the scatter. Year 0 is the average of the sunspot minimum years 1934, 1944, 1954 and 1965. The effect of uncertainties in the computation of the magnitude of the Rosenberg-Coleman effect will usually be to decrease the amplitude of the effect and, therefore, the latitude values shown in Fig. 3 should be considered upper limits. We may expect considerable variation from the average effect shown in Fig. 3, and in particular for intervals of several months near sunspot minimum the current sheet may occupy only a few degrees of latitude.

As a first approximation, we assume that galactic cosmic rays have relatively difficult access to the inner Solar System in the portion of the heliosphere occupied by the changing fields of the sector structure, and relatively easy access through

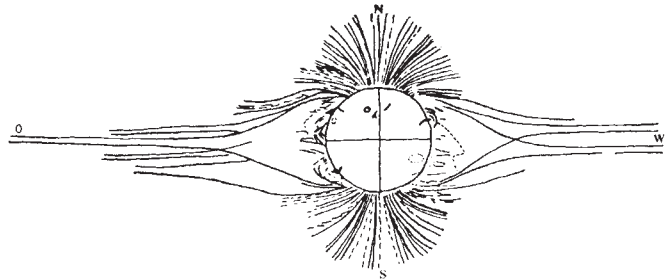


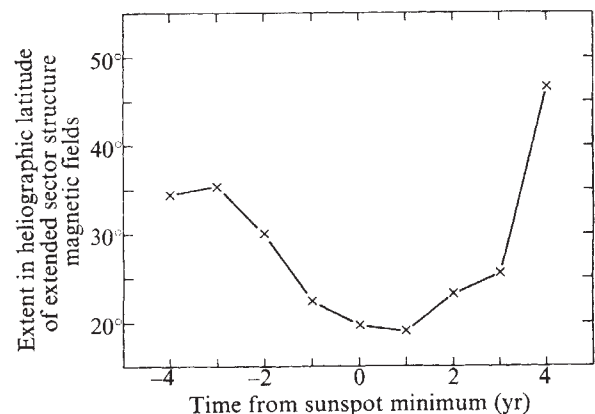
Fig. 2 The structure of a sunspot minimum solar corona drawn from eclipse photographs¹¹ (June 30, 1954) obtained in Kozelsk.

the portions of the heliosphere occupied by the extended uniform solar polar fields. This assumption is consistent with the observational evidence now available. A final evaluation must await *in situ* observations with out-of-ecliptic spacecraft. It is also observed⁸ that high speed solar wind streams are contained within sectors. Kinks and irregularities in the interplanetary magnetic field that can scatter cosmic rays are produced as the fast plasma in a high speed stream overtakes slower plasma. The solar-wind plasma coming from higher solar latitudes may have a more uniform (albeit larger) velocity than the solar wind plasma from lower solar latitudes, and thus be not as effective in producing scattering of cosmic rays.

In Fig. 4 we show the solid angle of the heliosphere occupied by the extended solar polar fields through an average sunspot cycle, where the latitude angles shown in Fig. 3 are used to compute the solid angles shown in Fig. 4. Also shown in Fig. 4 are the monthly averages of the absolute intensity of primary cosmic rays of rigidity > 0.5 GV observed near Murmansk and at Mirny from 1958 to 1973 (ref. 19). The total flux of such galactic cosmic rays in the interstellar medium in the vicinity of the Earth is estimated to be $4,000 \text{ m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ (ref. 19). Therefore, in Fig. 4 we have set the total solid angle 4π of the heliosphere as equivalent to a flux of $4,000 \text{ m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$, and the zero of the solid angle scale corresponds to zero flux. This corresponds to the assumption that galactic cosmic rays have easy access to the inner Solar System through the solid angle of the heliosphere occupied by the extended solar polar fields, and difficult access through the solid angle occupied by the sector structure fields.

In Fig. 4 we see that the average sunspot cycle variation of the solid angle of the extended solar polar fields is rather similar to the observed variation of the flux of primary cosmic rays of rigidity > 0.5 GV between 1961 and 1969. We should not

Fig. 3 Computed variation of the average extent in heliographic latitude of the extended solar sector magnetic fields. Year 0 is the average of the sunspot minimum years 1934, 1944, 1954 and 1965. The value 20° on the ordinate means that the extended sector fields are in the interval 20°N to 20°S .



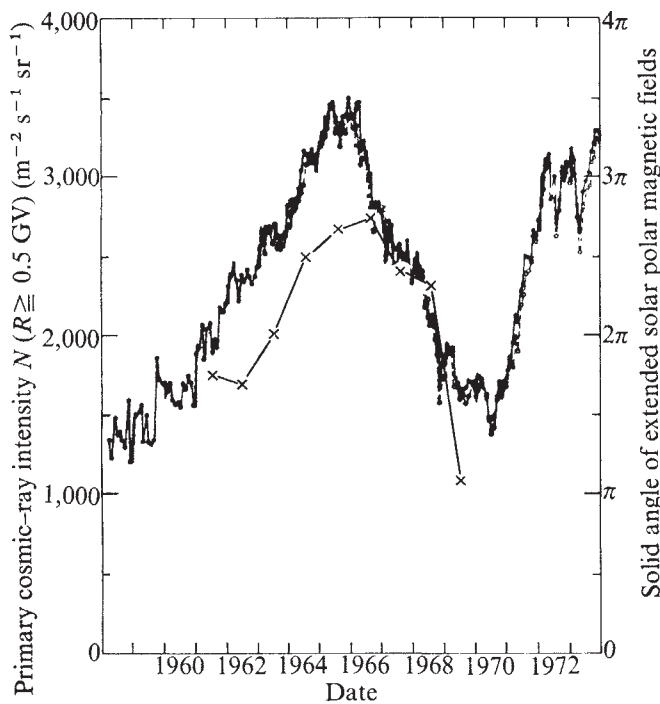


Fig. 4 Galactic cosmic-ray intensities with rigidity 0.5 GV observed near Murmask (●) and at Mirny (○) from 1958 to 1973 (ref. 19). Also shown is the computed average solid angle (×) of the heliosphere occupied by the extended solar polar magnetic fields.

expect a detailed agreement between the computed variation of solid angle averaged over four sunspot cycles and the observed cosmic-ray flux around a single sunspot minimum. The similarity of the two curves in Fig. 4 suggests that there may be some validity to the considerations advanced in this paper. The detailed computation of the diffusion lengths of cosmic rays related to these considerations is beyond the scope of this paper.

We thank the NASA-Skylab Workshop on Coronal Holes for discussions. This work was supported in part by the Office of Naval Research, by NASA, by the Atmospheric Sciences Section of the NSF and by the Max C. Fleischmann Foundation.

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Received March 22; accepted July 5, 1976.

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Glass-rich basaltic sand and gravel within the oceanic crust at 22°N

DURING Leg 46 of the Deep Sea Drilling Project (January 29–March 10, 1976), the second leg of the International Phase of Oceanic Drilling, the Glomar Challenger drilled into 255 m of basaltic basement at hole 396B, 160-km east of the Mid-Atlantic Ridge valley at 22°59.14'N, 43°30.90'W. The hole was drilled into crust with a magnetic anomaly age of ~ 10 Myr. This site is located in a N–S trending sediment pond which measures ~ 4 km × 10 km and is concordant with local topographic trends. There are 150 m of sediment at the drill site; however, the basement slopes regionally to the east and sediment thickness is probably > 250 m along the fault-bounded (?) eastern margin (G. M. Purdy, unpublished, and report of work by the Soviet research vessel Akademik Kurchatov). The bottom 90 m of this hole contained basaltic sand and gravel. This material is not believed to be a drilling artefact, but caused by *in situ* spalling or brecciation of basaltic pillow rinds.

On a number of other occasions, for example, on Legs 34 and 45, there was some indication, principally the sticking or jamming of the drill bit within young basement, that clastic zones had been encountered. At hole 396B a zone of rapid penetration was followed by an interval of difficult drilling and frequent sticking of the bit which eventually led to termination of drilling. In the lower part of the hole, the core catcher assembly was modified to catch fine clastic material and in cores 30 and 33, the core barrel contained 82 and 90 cm respectively, of basaltic gravel and sand. The recovery rate immediately above these two intervals was < 1% and on the basis of this and other shipboard data, in particular results from a unique downhole logging programme, we believe that the clastic zone was first encountered at 310 m sub-bottom and was apparently continuous down to at least 405.5 m where the hole was abandoned.

Stratigraphic units within 396B were determined on the basis of macroscopic and microscopic lithologies, magnetic properties, chemical composition, alteration features and density of recovered samples. In addition, we have been able to correlate these units with downhole logging records which include sonic velocity, natural γ -ray intensity, porosity, density and electrical conductivity. Figure 1 summarises the general lithologies, magnetic inclination, chemical stratigraphy (typified by TiO₂ concentrations) and dual induction lateral (conductivity) log. All indicators show a marked change in character below the 310-m depth where samples of the clastic material were first recovered.

The gravel and sand from cores 30 and 33 are largely in the coarse sand to fine gravel size range (0.5–3.0 mm) and are moderately sorted. The sorting, however, may be the result of preferential loss of fine grained material during core recovery. The fragments are generally non-vesicular and consist of angular chips of glass and variolitic, cryptocrystalline and intersertal basalt. Olivine and plagioclase occur as phenocrysts in the clasts and as crystal fragments. A small number of pieces of calcite-cemented basaltic microbreccia, a few chips with calcite spherules and clasts with cross-cutting calcite veins were found. In general, the glass appears quite fresh, palagonite rims are uncommon and few palagonite fragments were found.

Two small fragments (5–10 cm) of bedded hyaloclastite were also recovered in core 30. One of these samples is a moderately indurated fine to coarse grained hyaloclastite sandstone with graded bedding and evidence for scouring. Both rocks offer clear evidence for some sort of current action and the redistribution of dominantly sand-sized fragments of sideromelane and fine grained basalt.

The last 90 m, which we have called the clastic zone, is divided into four units: an upper clastic breccia, an upper basaltic gravel, a plagioclase phyric pillow basalt (breccia?) and a lower gravel unit. The two gravel units seem to have been derived locally. Crystalline fragments in the upper gravel are

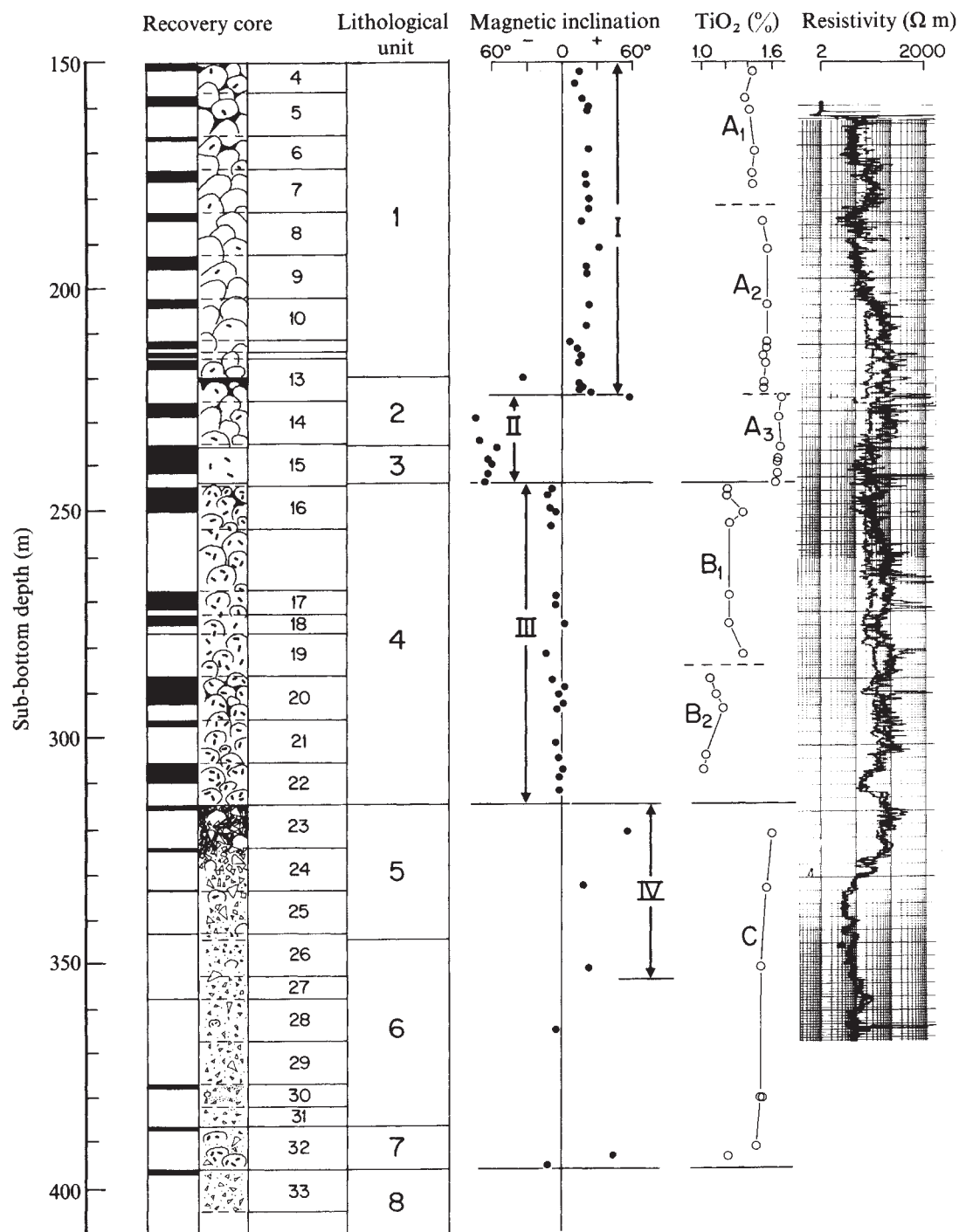


Fig. 1 Variations of lithological units, magnetic inclination, TiO_2 , and electrical resistivity log as a function of depth below the sea floor for hole 396B.

dominantly olivine-plagioclase phyrlic with proportions of olivine to plagioclase (ol.-plag. = 0.55) similar to the basalt in the overlying breccia unit, but unlike the basalt cored previously above the clastic zone. The lower gravel unit, on the other hand, contains predominantly plagioclase phyrlic crystalline fragments (ol.-plag. = 0.13) similar to the intervening plagioclase phyrlic pillow basalt (breccia?).

Four major processes may be invoked to explain the fragmentation of the basalt:

(1) Fragmentation during eruption and cooling: this may involve several interrelated processes peculiar to submarine lava flows. (a) Glassy fragments could form by granulation due to thermal shock resulting from quenching of lava by seawater. (b) Spalling and crumbling of pillow rinds as a result of continued or multiple flow of basaltic lava causing inflation

of a pre-existing pillow crust. (c) Brecciation from the oversteepening of pillow mounds causing them to tumble or slump. Mechanical shock from earthquakes or other tectonic processes may facilitate in this type of break-up.

(2) Tectonic brecciation after consolidation. Faulting and fissuring within the median valley, and on the flanks and away from the ridge is ubiquitous.

(3) Erosion, transport and deposition including the formation of talus slopes associated with fault scarps. Erosion would probably be largely restricted to reworking of material previously broken by processes 1 and 2.

(4) Shaving or reaming out of the hole by the drill bit during re-entry or fragmentation by drilling.

Origin 4 is unlikely: no re-entry was made immediately before coring the fragmented debris; the penetration rates

were exceptionally high; lithified sandstone fragments were found with the loose gravel and sand; the lithic clasts do not seem to have been derived from higher in the bore hole and the data from the downhole logs strongly suggests that the clastic material represents a distinct lithological unit. The abundance of fragments of basaltic glass in the clastic material indicates that much or all of it is derived from basaltic pillows; however, as the majority of the fragments are partly crystallised (origin 1(a)), spallation of glass rinds on quenching, would seem to be ruled out as a major source for the clastic material. The calcite fragments, spherulites, clasts with cross-cutting calcite veins and calcite cemented basaltic microbreccia fragments demonstrate that some of the gravel and sand must be late clastic debris from previously altered flows. On the basis of available data, however, it is impossible to make a clear choice between origins 1(b), 1(c), 2 and 3 as the predominant source of the gravel sand and other clastic debris in the clastic zone.

Although deposits of coarse talus have been well known for many years from photographs of the sea floor we believe that this is the first time that a deposit of relatively fine grained clastic material has been recovered from beneath the sea floor, and it represents a hitherto unknown element among these rocks. We have provided evidence that it comprises the greater part of the lower 90 m of the core, but may extend deeper. Since this material cannot be strongly magnetised and cannot contribute to the lineated magnetic anomalies of this region, it probably does not make up a significant portion of the basement.

We wish to thank Captain Lloyd E. Dill and his ship's crew, Cotton Guess, Drilling Superintendent, and his drilling crew, Robert Knapp, Operations Manager, G. Bode and the DSDP technicians and Dalhousie University, CNEXO, Schlumberger Well Services for supplying major equipment and services. The International Programme of Ocean Drilling is funded by the governments of England, France, Germany, Japan, the United States and the USSR through the NSF and managed by the Scripps Institution of Oceanography.

SCIENTIFIC PARTY DEEP SEA DRILLING PROJECT, LEG 46*

Received May 17; accepted July 8, 1976.

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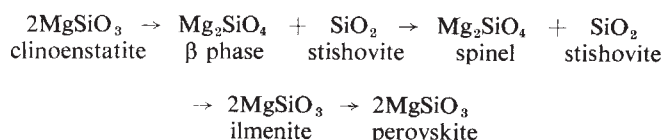
The post-spinel phase of forsterite

THE nature of the post-spinel phase of forsterite (the olivine structure of Mg_2SiO_4) and its importance to our understanding of the mineralogy, petrology and seismological discontinuities in the transition zone and the lower mantle of the Earth has attracted much recent attention^{1,2}. Kumazawa *et al.*¹ reported that the post-spinel phase of forsterite recovered from 3.3×10^{10} Pa and $1,000^\circ\text{C}$ was a component oxide mixture of stishovite (SiO_2) plus periclase (MgO). In their studies of Mg_2SiO_4 - Fe_2SiO_4 solid solutions at pressures up to 2.5×10^{10} Pa, Ming and Bassett³ agreed with the conclusions of Kumazawa *et al.* Consequently, the oxide mixture seems to be widely accepted as the post-spinel phase of forsterite³. In this paper, however, I present results that disagree with this.

I have previously found that forsterite transforms into an assemblage of perovskite (MgSiO_3) plus periclase (MgO) when subjected to pressures $> 3.0 \times 10^{10}$ Pa and $\sim 1,000^\circ\text{C}$ (ref. 4). Since the zero pressure density for the perovskite + periclase assemblage is 1.9% greater than that of the mixed oxides,

I have designated this assemblage as the post-oxide phase of forsterite and have postulated that the Earth's lower mantle comprises mainly the perovskite phase of MgSiO_3 (ref. 5).

Until my work on the detailed high pressure phase transformations in MgSiO_3 (ref. 6), I had not doubted the interpretation of the post-spinel phase for Mg_2SiO_4 as representing mixed oxides. A schematic isothermal phase diagram for the system MgO-SiO_2 at $1,000^\circ\text{C}$ is shown in Fig. 1. If Mg_2SiO_4 were to disproportionate into its component oxide mixture at certain pressure and temperature conditions, so also should MgSiO_3 . The latter, in fact, has been reported by Ming and Bassett⁷ who have found that MgSiO_3 disproportionates into its component oxides, stishovite plus periclase, at a pressure of $\sim 2.5 \times 10^{10}$ Pa. In my recent study of the high pressure phases of MgSiO_3 between 1.5×10^{10} - 3.0×10^{10} Pa loading pressure⁶, however, I have found that the sequence of phase transformations in MgSiO_3 with increasing pressure is as follows



If there were a stability field for the mixed oxides, it would have intervened between the ilmenite and the perovskite phases.

The ilmenite phase of MgSiO_3 transforms directly into the perovskite phase with increasing pressure. This behaviour is consistent with phase transformations observed in all the available ilmenite analogues, CdTiO_3 (ref. 8), CdSnO_3 (ref. 8), MnVO_3 (ref. 9), $(\text{Fe,Mg})\text{TiO}_3$ (ref. 10), MnGeO_3 (ref. 11), and MgGeO_3 (L.L., unpublished). With the exception of ZnSiO_3 , for which only a very small stability field of mixed oxides has been reported¹², the ilmenite phase of all the above-mentioned compounds has been found to transform directly into the

Fig. 1 Schematic isothermal phase diagram for the system MgO-SiO_2 at $1,000^\circ\text{C}$.

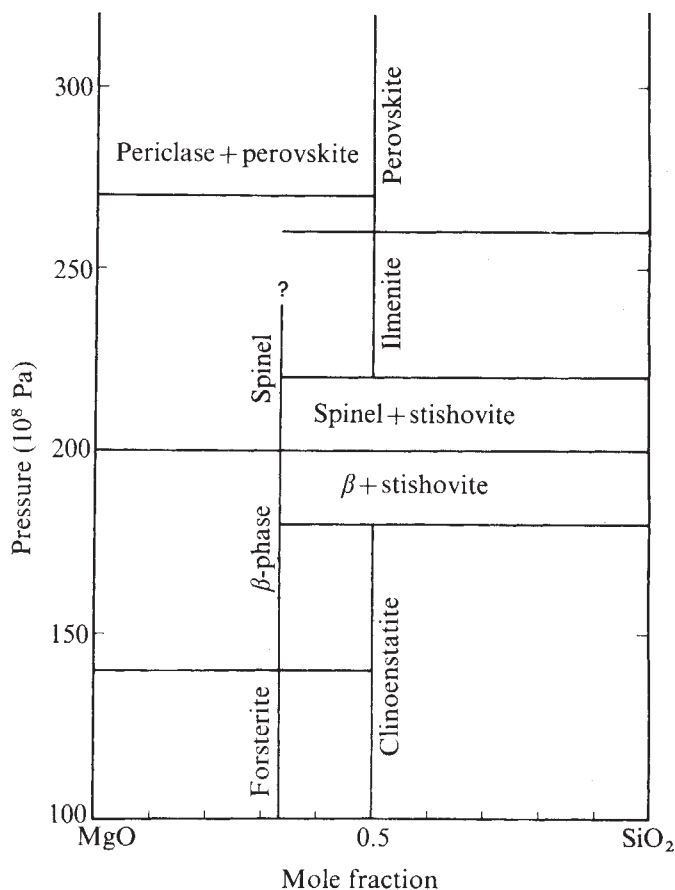


Table 1 X-ray diffraction data (room temperature and 10^5 Pa pressure) for forsterite quenched from a loading pressure of $\sim 2.2 \times 10^{10}$ Pa and temperature of $\sim 1,000^\circ\text{C}$ (CoK α)

Observed*			Forsterite†			Spinel‡			Perovskite§			Periclase†			Stishovite¶		
<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀		<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀	<i>hkl</i>	<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀	<i>hkl</i>	<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀	<i>hkl</i>	<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀	<i>hkl</i>	<i>d</i> (Å)	<i>I</i> / <i>I</i> ₁₀₀	<i>hkl</i>
5.12	10		5.11	26	020												
3.90	25		3.88	69	021												
3.74	20		3.73	25	101												
3.51	10		3.50	10	111												
3.45	25								3.45	60	002						
3.09	15								3.08	15	111						
3.00	20		3.00	17	121										2.955	100	110
2.863	35					2.855	23	220									
2.774	25		2.768	53	130												
2.520	25		2.513	73	131												
2.445	100b		2.458	100	112				2.471	20	020	2.431	10	111			
2.396	15					2.435	100	311	2.435	100	112						
2.333	10					2.331	5	222	2.395	20	200						
2.263	20		2.268	59	122												
2.199	5								2.196	5	120				2.247	25	101
2.161	20		2.161	15	211												
2.110	50											2.106	100	200			
2.081	10								2.085	30	013						
2.059	15								2.057	25	211						
2.025	40					2.019	50	400									
1.970	10								1.967	10	202				1.979	40	111
1.915	15								1.911	25	113						
1.884	5		1.878	5	150										1.869	20	210
1.856	15								1.853	15	122						
1.830	5								1.828	5	212						
1.752	30		1.748	60	222												
1.723	40								1.724		004						
1.688	5								1.720	80	220						
1.671	5		1.670	13	241				1.683	10	023						
1.652	10					1.649	9	422	1.669	10	221						
1.575	5		1.572	10	043												
1.558	35					1.554	25	333									
1.541	5								1.539	15	222						
1.521	15														1.530	60	211
1.493	30		1.497	27	004				1.520	20	131						
1.484	5								1.484	5	311						
1.430	45					1.428	63	440							1.478	25	220
1.418	10								1.420	40	132						
1.403	10								1.399	10	204						
1.392	25		1.394	9	233				1.390	45	312						
1.352	5		1.351	17	322												
															1.332	9	002
															1.322	4	310
1.317	5		1.316	9	134										1.234	35	301
1.232	15					1.232	10	533							1.215	10	112
1.218	25								1.216	15	041	1.216	12	222			
1.195	5								1.197	5	140						
1.167	10		1.166	2	—	1.166	6	444	1.164	10	410				1.184	3	311
1.155	<5		1.155	1	352				1.158	5	323				1.159	7	320
1.092	5		1.099	1	412				1.098	15	240						
1.077	10					1.079	5	642	1.078	10	420						
1.051	20					1.051	12	553									
1.035	<5		1.036	3	334							1.053	5	400			
1.021	<5		1.020	3	0,10,0												
1.009	10					1.009	7	800									
6 extremely weak lines																	
0.9417	10											0.9419	17	420			

* With the exception of film shrinkage, no other corrections to the data have been made.

Values for *I*/*I*₁₀₀ were estimated visually. *b*, Broad line.

† From ASTM Card Catalogue (4-0768 and 4-0769) for forsterite and (4-0829) for periclase.

‡ From ref. 15.

§ From ref. 4.

¶ From ref. 16.

perovskite phase without the intervention of any other phases.

The fact that the ilmenite phase of MgSiO_3 transforms directly into the perovskite phase limits the post-spinel phase for Mg_2SiO_4 to the following possibilities: (1) a single denser phase of Mg_2SiO_4 ; (2) a mixture of ilmenite (MgSiO_3) plus periclase (MgO); (3) a mixture of perovskite (MgSiO_3) plus periclase; or (4) any combination of them (see Fig. 1). The second possibility has been previously postulated¹².

I have made 12 experimental runs on forsterite in the loading

pressure region between 1.5×10^{10} and 3.2×10^{10} Pa and temperatures ~ 900 – $1,200^\circ\text{C}$. The starting material is a synthetic forsterite (Mg_2SiO_4) powder (Tem-pres Research Inc.). The fine polycrystalline sample was mixed intimately with a small amount of powdered graphite, which serves to absorb laser radiation and to heat the sample under compression. The sample was then compressed in a diamond anvil press fitted with a lever and spring assembly and heated by the continuous YAG laser while the sample was maintained at pressure. The

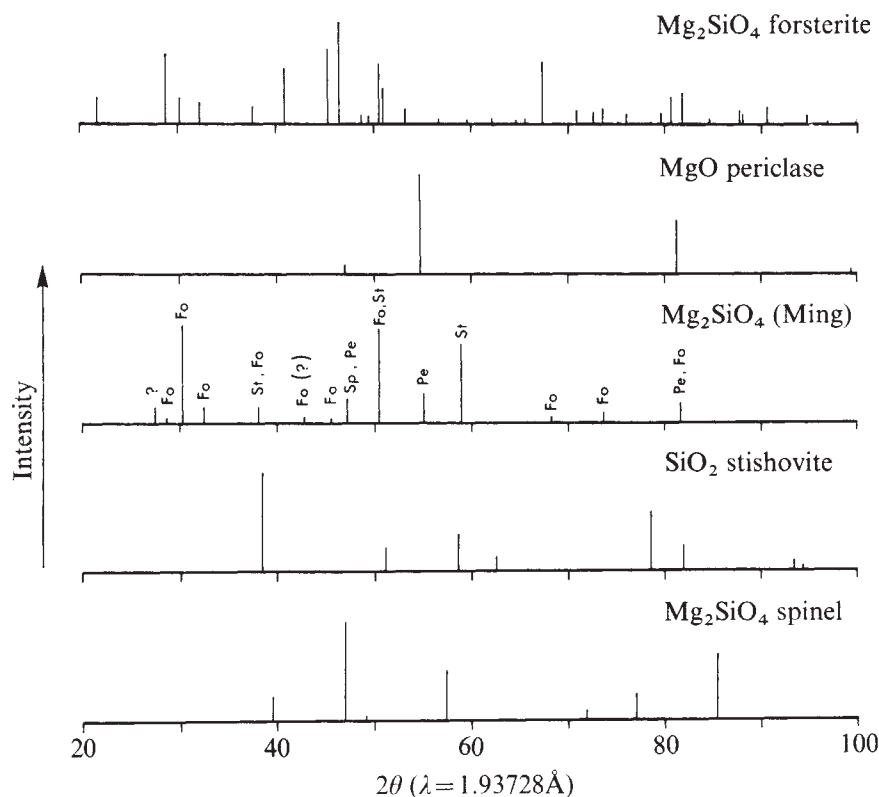


Fig. 2 X-ray diffraction data for forsterite quenched from $\sim 2.5 \times 10^{10}$ Pa and $1,400^\circ\text{C}$ (the temperature is highly uncertain) from ref. 14 in comparison with other relevant patterns. Fo, Forsterite; St, stishovite; Pe, periclase.

loading pressures of the sample at the central portion of the anvil were estimated from the length of the spring, and are probably accurate to $\pm 10\%$. Sample temperatures were estimated to be $\sim 1,000^\circ\text{C}$ with large uncertainty. After quenching and release of pressure, samples were transferred to a modified 57.3-mm Debye-Scherrer camera for X-ray diffraction study. Details of the procedure have been described elsewhere¹³.

In the pressure region between 1.7×10^{10} and 2.5×10^{10} Pa (inclusive), nine runs were made at 10^9 -Pa intervals; additional runs have been made at 1.5 , 2.8 and 3.2×10^{10} Pa. The spinel phase of Mg_2SiO_4 seems to transform directly into a mixture of perovskite (MgSiO_3) plus periclase (MgO) at $\sim 2 \times 10^{10}$ Pa. The results obtained at pressures $> 2 \times 10^{10}$ Pa showed a steady increase of the yield of the perovskite + periclase assemblage with increasing pressure (Table 1). No stishovite, that is, no component oxide mixture, has been identified in the entire pressure region between 1.5×10^{10} and 3.2×10^{10} Pa in this study. These results are in accordance with those of my previous studies on MgSiO_3 (ref. 6), but contrast markedly with those reported in refs 1 and 2.

Ming and Bassett² did not report the detailed experimental results for Mg_2SiO_4 in their paper, but these data can be found in Table 10 of ref. 14 and have been reproduced graphically here in Fig. 2, together with all the other related phases. In the pattern obtained by Ming, there are only three lines which were attributed to stishovite, but their relative intensities contrast greatly with those for the pattern of stishovite shown in Fig. 2. It is also very surprising that the second intense line of stishovite at $2\theta = 78.5^\circ$ was not observed in Ming's pattern.

Kumazawa *et al.*¹ reported that forsterite disproportionated into a mixture of stishovite plus periclase on the basis of a single run at 3.3×10^{10} Pa and $1,000^\circ\text{C}$ for 30 min. (Their pressure should be revised to $\sim 2.2 \times 10^{10}$ Pa and temperature should be much higher (Kumazawa, personal communication).) In none of the present experiments at loading pressures between 1.5×10^{10} and 3.2×10^{10} Pa did I observe the mixed oxide phase assemblage. It is unlikely that the differences in the experimental conditions of pressure and temperature can explain this apparent discrepancy between my results and theirs.

Further support for the present results on the post-spinel phase of Mg_2SiO_4 is provided by studies of the post-spinel phases for 12 silicate and germanate compounds with the olivine stoichiometry in this laboratory. At pressures $> 2.5 \times 10^{10}$ Pa, only Mg_2SiO_4 , Mg_2GeO_4 and Mn_2GeO_4 have been found to transform into the perovskite + rocksalt assemblage. The remainder transform into their component oxides with the rutile and rocksalt structures. The oxide mixture phase assemblage was not found as an intermediate phase between the spinel phase (or the β phase, which is a distorted spinel phase) and the perovskite + rocksalt assemblage in any of the above three compounds.

The associated increase in zero pressure density from the spinel phase of Mg_2SiO_4 to the mixture of perovskite (MgSiO_3) plus periclase (MgO) was calculated to be 10.7% . This spinel-post-spinel reaction is characterised by an increase in coordination number of oxygen for Si^{4+} from 4 to 6 and for half of the Mg^{2+} from 6 to 8.

I am grateful to A. E. Ringwood and R. C. Liebermann for comments.

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Received June 4; accepted July 7, 1976.

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Liquid nitrogen enhancement of partially annealed fission tracks in glass

THE number density of fission tracks in solids is reduced if the sample is heated before chemical etching, and the effect of annealing must be accounted for before an age date can be assigned to the sample¹⁻³. The extent of the annealing can be observed by a measurement of the reduction in the track parameters (diameter and/or length) and subsequent comparison to unannealed tracks. Correct ages can be obtained by careful calibration studies of track density reduction against track diameter or length reduction at different annealing temperatures and times^{4,5}. For crystalline minerals, however, these correction techniques are not generally valid⁶. In this study, glass samples were partially annealed and then immersed in liquid nitrogen for various periods and we show that the properties of the glass and/or the track parameters can be altered so as to observe tracks that would normally be erased by annealing.

An NBS glass standard (SRM-962) with latent fission tracks was cut in half with a diamond cutting wheel. The exterior surfaces were ground and polished with 12.5- μm and 0.3- μm alumina powder such that at least 30 μm was removed from the surface. The halves were etched simul-

taneously in 16% HF at room temperature for 75 s and rinsed in warm running water for several minutes. Etched tracks were observed and counted with a Leitz microscope at overall magnifications of 800 and 1,600. The $\times 800$ magnification was used to increase the counting statistics and the $\times 1,600$ was used to identify the fission tracks properly⁷. Track length and width measurements were made at $\times 1,600$ with an eyepiece reticule.

One of the samples was partially annealed at 230 °C for 1 h. Both halves were ground, polished and etched together as described previously. The track density of the partially annealed sample was found to be half that of the unannealed sample. The length and width of the partially annealed tracks were found to be similarly reduced.

Both samples were then immersed in liquid nitrogen for varying periods. At the conclusion of each immersion the

Fig. 1 Track density ratios as a function of liquid nitrogen immersion time. t/t' , Track density after immersion/track density at zero immersion time. ● Values found for unannealed sample; ■ values for partially annealed sample.

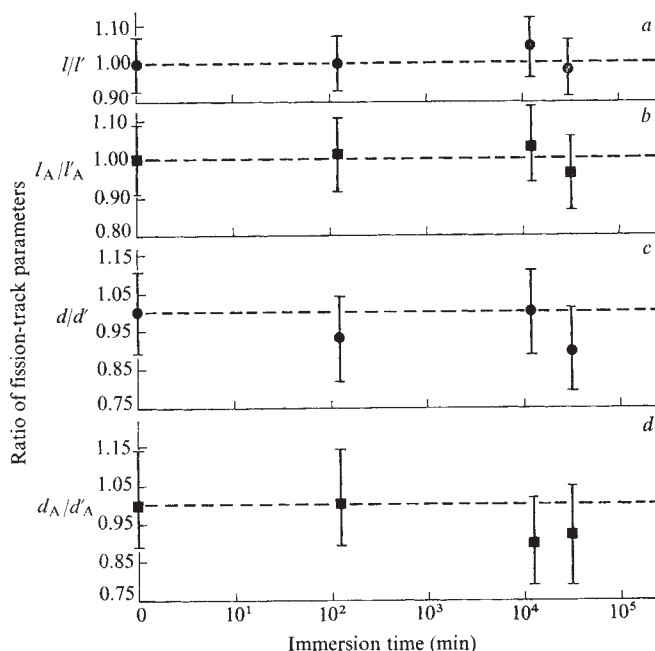
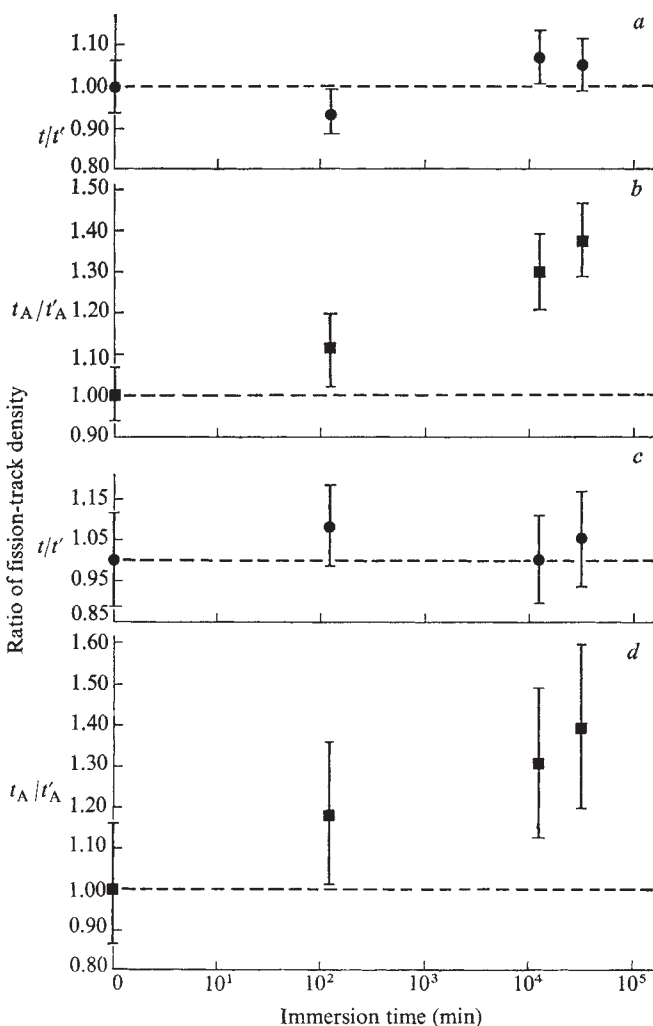


Fig. 2 Track parameter characteristics as a function of immersion time. l/l' , Length of track after immersion/length of track at zero immersion time; d/d' , diameter of track after immersion/diameter of track at zero immersion time. ●, Unannealed sample; ■, partially annealed sample.

samples were polished, etched and counted in the manner described above. The results of the track density measurements against liquid nitrogen immersion time are given in Fig. 1. To compensate for possible variations in etch conditions the ratios of track densities were determined by normalising with the track density having zero immersion time. Figure 1a and c shows the track ratios against immersion time for the unannealed sample at magnifications of 800 and 1,600 respectively. The ratio remains essentially constant about a value of one. Figure 1b and d shows the partially annealed track density increasing with immersion time for magnifications of 800 and 1,600 respectively. A gain of ~40% was achieved after 760 h of immersion in liquid nitrogen.

To ascertain whether the track parameters were changing with immersion time, the length and diameter of the tracks were measured after each etching at a magnification of 1,600. Figure 2 shows the ratio of length and diameter for the unannealed (2a, c) and the partially annealed (2b, d) samples. For each ratio, the measured quantity with zero immersion time was used to normalise the graphs. The size of the tracks was not noticeably affected by immersion in liquid nitrogen (Fig. 2).

It was thought that thermal shock (room temperature $\rightarrow$ liquid nitrogen temperature $\rightarrow$ room temperature) along

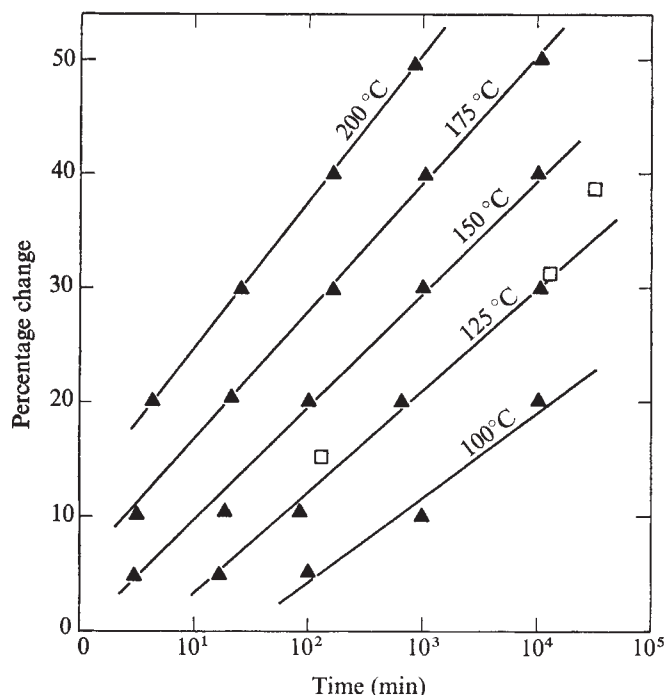


Fig. 3 Percentage change in track density against time for NBS glass standards. $\square$, Percentage track enhancement found in the present study; (—) connecting the points (Δ) indicate the percentage track reduction at different annealing temperatures (reported in ref. 8).

the latent track was the cause of the track enhancement. It was found, however, that repeated (four times) immersion of the samples in the liquid nitrogen (for 2 h) did not lead to an increase in the track density.

A recent study⁸ of the thermal stability of fission tracks in NBS glass standards reported a track density decrease with increasing annealing time and temperature. The percentage track reduction at different annealing temperatures and times from that investigation are replotted in Fig. 3, together with the average gain ($\times 800$ and $\times 1,600$ magnifications) against immersion time obtained in the present study. The slopes of the annealing plots and liquid nitrogen immersion plot seem to be compatible. This suggests that the mechanism which erases the track damage through annealing may be partially reversed when the temperature of the sample is significantly lowered for a sufficient length of time. Further work is under way (at different immersion temperatures and partial annealing conditions) to test whether or not the process of enhancement is a reversal of the annealing process. Similar enhancement effects using liquid nitrogen have been found for α -particle tracks in polycarbonate detectors and those results will be published.

Although the mechanism of track reduction with temperature rise (annealing) is not clearly understood⁷, track enhancement by cooling may provide a new avenue of research into this problem. In addition, this method has possible application in dating geological samples partially annealed by post-emplacement thermal events.

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Direct measurement of long range forces between two mica surfaces in aqueous KNO_3 solutions

WE have constructed an apparatus for measuring the forces between two surfaces immersed in a liquid directly as a function of separation, and report initial results for the forces between two crossed cylindrical sheets of molecularly smooth muscovite mica in aqueous solutions of KNO_3 .

The apparatus is shown schematically in Fig. 1. It is similar to earlier models used for measuring the van der Waals' forces between two freshly cleaved mica sheets in air *in vacuo*^{1,2}. The present apparatus has been refined to allow for the measurement of the very different types of forces in the conditions that occur in liquids. Multiple beam interferometry is used for measuring the shape of the surfaces and their separation to within 0.1 nm (ref. 3). The separation between the two surfaces is controlled by a three-stage mechanism: an upper micro-driven rod (to $\sim 1 \mu\text{m}$), lower micrometer-driven rod (to $\sim 1 \text{ nm}$) and a piezoelectric crystal (to $< 0.1 \text{ nm}$). The lower rod operates by a differential spring mechanism: the double cantilever spring is about a thousand times stiffer than the helical spring so that a $1\text{-}\mu\text{m}$ movement of the motor-driven lower rod is reduced to a 1-nm movement between the two mica surfaces. All parts of the apparatus are made of 316 stainless steel, Teflon, Delrin and glass. Extreme precautions were taken to purify the water and potassium nitrate, and a high degree of surface cleanliness was maintained in the equipment. The apparatus is suspended by springs to isolate it partially from extraneous vibrations. Some residual vibrations of the surface (a few Å) was found to be an asset, in that it indicated that the surfaces were truly separated and not in contact through a dust particle or a mica flake. In view of the repulsive forces measured, this precaution was essential, since a minute particle between the surfaces far from the region of closest approach could go undetected and lead to a spurious repulsion being measured.

In the present set-up the glass disk supporting the lower mica sheet was suspended at the end of a cantilever spring of stiffness $\sim 10^2 \text{ N m}^{-1}$. The forces were measured by applying a sudden voltage to the piezoelectric crystal, which causes it to expand or contract by a known amount, say 10 nm. The resulting change in the separation between the two surfaces is then measured optically and any difference in the two values, when multiplied by the stiffness of the single cantilever spring, gives the force difference, whether attractive or repulsive, between the initial and final separations. In this way, forces may be measured at any separation down to contact.

Figure 2 shows some of these results for mica in KNO_3 solutions at 20°C , $\text{pH} \sim 6$. The repulsive forces were all found to be exponential, with a decay length close to the Debye length. Below a certain separation, however, the repulsion increased more rapidly. The onset of this enhanced repulsion occurred very roughly at about half a Debye length separation. The magnitude of the repulsive forces was found to be different for the different mica samples used. In 0.1 M and 1 M solutions the onset of repulsion was preceded by an attractive region for those samples in which the repulsion was weak, thereby indicating the existence of a secondary minimum at these concentrations. It

Received May 12; accepted July 12, 1976.

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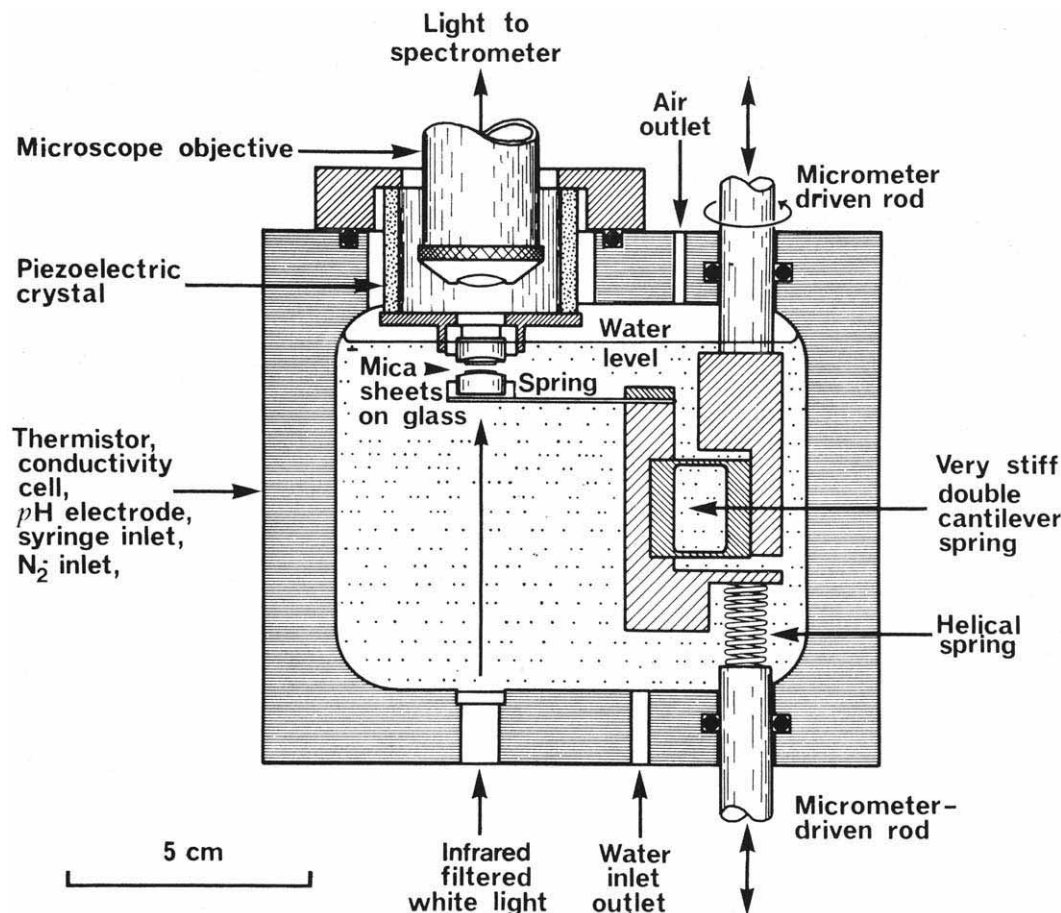


Fig. 1 Schematic drawing of apparatus to measure long range forces between two crossed cylindrical sheets of mica (of thickness $\sim 1 \mu\text{m}$ and radius of curvature $\sim 1 \text{ cm}$) immersed in liquid. By use of white light and multiple beam interferometry the separation between the two mica surfaces may be measured to $\sim 0.1 \text{ nm}$. The separation between the two mica surfaces may be controlled by use of two micrometer-driven rods and a piezoelectric crystal to better than 0.1 nm .

was also observed that the repulsion was irreversibly reduced after the surfaces had been brought very close together ($<4 \text{ nm}$), reminiscent of qualitatively similar observations on aqueous dispersions of sodium montmorillonite sheets in NaCl solutions^{4,5}. At larger separations, however, the results were reversible and reproducible and clearly exhibit all the essential features of a DLVO-type interaction involving repulsive double layer forces and attractive van der Waals' forces^{6,7}. In particular, we note that even in 0.1 M KNO_3 the decay length of the exponential repulsive region is equal to the Debye length ($\sim 1 \text{ nm}$) indicating that the Poisson-Boltzmann equation, at least for KNO_3 , seems to remain valid even at this high concentration. The observation that at separations smaller than the Debye length the repulsive forces increased even more rapidly is qualitatively consistent with theoretical predictions of double-layer forces based on the nonlinear Poisson-Boltzmann equation⁸. The magnitude of the attractive forces in the region of the secondary minimum is of the order to be expected from the general theory of van der Waals' forces.

It is difficult to say without more detailed analysis, whether at very small separations, $<1 \text{ nm}$, there is also a primary minimum, since at these separations the mica sheets have deformed appreciably under the influence of the strong forces. Tearing of mica was, however, often observed on separation after very close approach, thus indirectly pointing to the existence of strong adhesion at or near contact. The lack of a clear-cut turnover in the force into a primary minimum is consistent with previous measurements on the interactions between other silicate

sheets^{4,5}, as well as the observation that silica dispersions are sometimes stable at very high electrolyte concentrations and undergo reversible coagulation⁹.

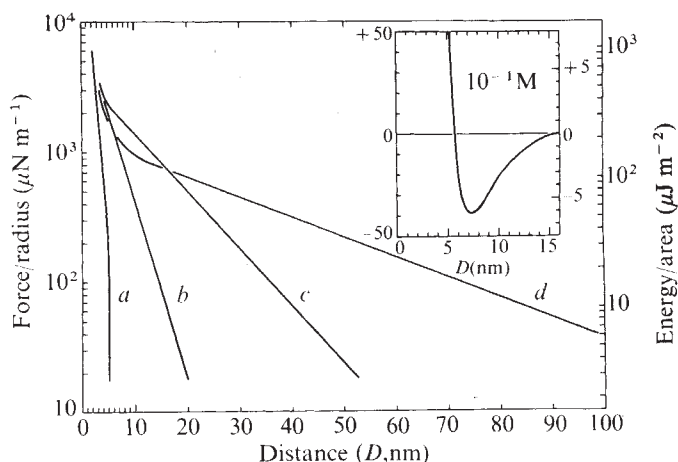


Fig. 2 Experimental results of direct measurement of force as a function of separation between two crossed mica cylinders of radius R in various KNO_3 solutions. The right-hand ordinate gives the interaction energy per unit area for two parallel plates, calculated according to the 'Derjaguin approximation'⁷. The ordinate of the inset is linear. The magnitude of the repulsive forces was found to be different for different mica samples. The results shown here are for those samples in which the repulsion was relatively weak and where a secondary minimum was clearly observed. The scatter in the experimental points is $\sim 10 \mu\text{N m}^{-1}$ for the force/radius. *a*, 10^{-1} M ; *b*, 10^{-2} M ; *c*, 10^{-3} M ; *d*, 10^{-4} M .

We thank Dr T. W. Healey, Professors R. J. Hunter and B. W. Ninham for helpful discussions, Mr P. East for machining, and Mr J. Gascoigne for technical assistance.

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Received June 23; accepted July 9, 1976.

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Axial, magnetron, cyclotron and spin-cyclotron-beat frequencies measured on single electron almost at rest in free space (geonium)

THE mono-electron oscillator¹ consists of a ~ 1 -meV electron contained in a weak electric quadrupole field plus strong axial magnetic field (the Penning trap) on which the axial oscillation is continuously observable. It may profitably be looked on as an ultraheavy metastable pseudoatom, 'geonium' (through the trap and the magnet, the electron is bound to the Earth ultimately). The energy levels are given by²

$$h^{-1}E_{mnkq} = mv_s + (n + \frac{1}{2})v_c + (k + \frac{1}{2})v_z - (q + \frac{1}{2})v_m$$

$$m = \pm \frac{1}{2}; n = 0, 1, 2, 3, \dots; k = 0, 1, 2, 3, \dots; q = 0, 1, 2, 3, \dots$$

with $v_c = eH/2\pi mc$, $2v_c v_m - 2v_m^2 = v_z^2$. We report here the measurement of the axial, magnetron, cyclotron, and spin-cyclotron-beat frequencies v_z , v_m , $v_c - v_m$ and $v_s - v_c + v_m$ using this apparatus, held at 4 K. A new axial-frequency-shift technique using a 30-gauss deep magnetic bottle superimposed on the 18-kgauss field supplied by a superconducting magnet was used for the last two frequencies. The road to this advance was paved by two developments, namely the anharmonicity

Fig. 1 First spin flip seen in the mono-electron oscillator (geonium). Because of the random fluctuations in the thermally excited cyclotron motion, the axial frequency shift δv_z associated with the magnetic bottle shows a corresponding unsymmetric fluctuation always staying above a fixed floor for a given spin direction. This floor, however, suddenly changes by 2.5 Hz when the spin is flipped, which occurs occasionally near resonance, $v_z - v_d \approx 2$ kHz. No auxiliary drive was used. Three superimposed traces are seen, only the heavy one showing the spin flip.

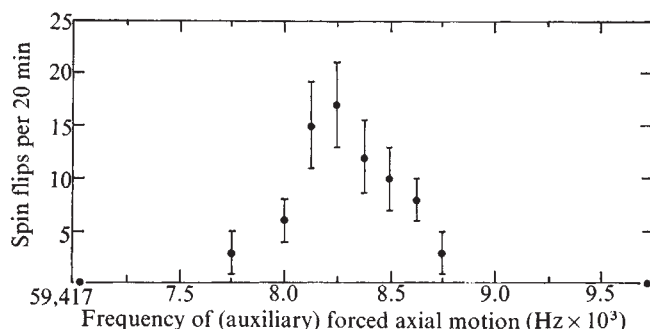
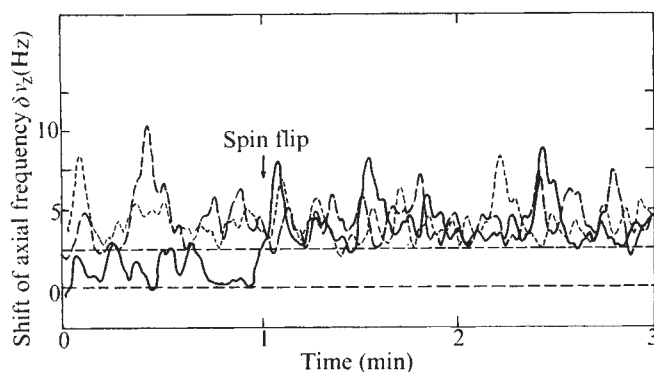


Fig. 2 Resonance at the spin-cyclotron-beat frequency v_d in geonium. The auxiliary axial drive amplitude has been adjusted thus, that spin flips occur no faster than 1 min^{-1} , and can be conveniently counted in 20-min periods, during which the auxiliary drive frequency is kept constant. Data from a later run are shown.

compensated trap³ yielding an axial line width of 10^{-7} and the "entropy reduction by motional sideband excitation" (ref. 4) method. The latter allows the centring of an electron liberated in the trap to close tolerance guaranteeing the constancy of v_z .

Essentially as proposed by us⁵⁻⁷, the resonance at $v_d = v_s - v_c + v_m$ was observed by making the electron see an effective, adjustable ($< 10 \mu\text{gauss}$) rotating magnetic field at v_s , the spin frequency, occurring as the sum of an (auxiliary) forced axial motion frequency $v_d = 59.415890$ MHz and $v_c - v_m \approx 51206.11$ MHz, the frequency of the thermal cyclotron motion through the inhomogeneous static field of the magnetic bottle. Spin flips, thereby induced at a rate of 1 min^{-1} , were detected by continuously monitoring the bottle-related shift $\delta v_z \approx (m + n + \frac{1}{2}) 2.5$ Hz of the axial resonant frequency $v_z = 59.4101$ MHz and focusing on the minimum values, 2.5 and 0 Hz, corresponding to $n = 0, m = \pm \frac{1}{2}$, which occur as the electron jumps frequently between the cyclotron levels, n , and rarely between the spin levels, m (Fig. 1).

The minimum v_d linewidth of ~ 500 Hz realised so far (see Fig. 2), arises primarily from the continuous v_z monitoring. Pulsed operation promises a 100-fold sharper line. With the measured $v_m = 34.464$ kHz, our preliminary value of $a \equiv (v_s - v_c)/v_c$ is 0.001159655(5). This agrees with the theoretical value⁶ for $(g-2)/2$ quoted with an error ten times smaller. The empirical dimensionless ratio a can contain information only on the structure of the electron. Just as in an atom the experimental g -factor provides an important check on the 'purity' of an actual stationary state when expanded after states of an approximate Hamiltonian, thus a is a measure of the contribution to the actual electron wave function by other elementary particles which, together with the Dirac electron, may all be considered as stationary states of the same approximate Hamiltonian describing the spectrum of matter-energy. As a consequence, useful bounds are put on weak interaction models⁹.

D. Wineland and P. Schwingberg have made important contributions to the work. Critical comments on the experiment by D. G. Boulware, L. S. Brown, P. Franken, O. Haxel, E. M. Henley, W. E. Lamb, Jr., R. Peierls, E. M. Purcell, E. Teller, L. Willets, and R. W. Williams are acknowledged by H. D. We thank the NSF for support.

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Entropy reduction by motional sideband excitation

EVERYONE is intuitively familiar with the second law of thermodynamics: the disorder in any closed system can only increase with time. A familiar example is the spreading of a scent through a room. It is also well known that the spreading greatly accelerates when exterior agents are at work, for example, a stirrer. The reverse task of 'bottling up' the diffused scent back in the flask from which it expanded is much more difficult. So if one had somehow succeeded in collecting a cloud of particles in a vacuum at the bottom of a suitable potential well, as would be desirable in removing Doppler shifts in coherent spectroscopy or even in nuclear fusion machines, there will be all sorts of effects at work to spread the particles out again. The principle of entropy reduction by motional sideband excitation¹ could be effective in re-collecting the spread out cloud at the bottom of the well.

As a specific example, consider an atom emitting optical radiation at ν_1 while vibrating in a translational mode at a radio-frequency ν_v in a parabolic well. Because of the Doppler effect, the optical radiation will then be frequency modulated at the radiofrequency ν_v . This implies that its spectrum will consist of a carrier at ν_1 and symmetric sidebands spaced at the radiofrequency ν_v . Inversely, if the vibrating atom is irradiated at one of the spectral optical components, say at the one at $\nu_1 - \nu_v$, it will be optically excited and will in turn radiate symmetrically at all the frequencies ν_1 , $\nu_1 \pm \nu_v$, $\nu_1 \pm 2\nu_v$, So, while absorbing quanta of energy $h(\nu_1 - \nu_v)$ it will radiate quanta of average energy $h\nu_1$, that is, larger by $h\nu_v$. This incremental energy can only come from the translational vibrational motion in the well, which is thereby reduced, bringing the atom closer to the centre of the well and possibly completely freezing out the quantised vibration. The principle is equally effective if the sidebands are created by amplitude modulation caused by motion through an inhomogeneous alternating field. The latter effect has, in fact, recently been demonstrated on a single electron confined in a Penning trap in ultra high vacuum. In such an axially symmetric trap, the electron is axially contained by an electric parabolic well while the corresponding radial potential hill is effectively overcompensated by an axial magnetic field. The guiding centre of the small diameter cyclotron orbit carries out a circular magnetron motion at $\nu_m \approx \nu_z^2/2\nu_c \approx 80$ kHz for an axial frequency $\nu_z \approx 60$ MHz and a cyclotron frequency $\nu_c \approx 20$ GHz. When the moving electron is now subjected to an axial r.f. field at ν_{zd} varying along the magnetron orbit, the electron will see spectral components at ν_{zd} , $\nu_{zd} \pm \nu_m$, $\nu_{zd} \pm 2\nu_m$. If one now chooses $\nu_{zd} = \nu_z + \nu_m$ the damped axial oscillation at ν_z will be excited. Hereby the energy excess $h\nu_m$ per absorbed $h\nu_{zd}$ -quantum will be used to move the electron up the radial electric potential hill until it reaches the axis. This effect and also the inverse one, namely rapid increase of the magnetron motion radius for excitation at $\nu_{zd} = \nu_z - \nu_m$, has recently been demonstrated by Van Dyck *et al.*² Monitoring the radial coordinate, r , of the electron was made possible by the presence of a small anharmonic term in the electric trapping potential which made ν_z a weak function of r .

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The low frequency dielectric constant of supercooled water

THE extension of computer dynamical calculations of water structure¹ to temperatures $< 0^\circ\text{C}$ has encouraged speculation that the supercooled liquid might exhibit ferroelectricity. The discovery that, by use of surfactants such as sorbitan stearates, water can be emulsified² in hydrocarbon solvents and then readily supercooled, opens up extensive new fields of experimentation. We have measured at 1,652 Hz the real part of the low frequency permittivity of 0.5% and 1% water emulsions down to -36°C and thence calculated the real part of the dielectric constant of liquid water, which is essentially static in this range of frequency and temperature. The values, shown graphically in Fig. 1, lie on a smooth curve and compare with data for polycrystalline ice³; no ferroelectric transitions are observed.

Emulsions of deionised water were made in *n*-hexane using as emulsifier polyoxyethylene sorbitan tristearate (Tweens 65, from Honeywell-Atlas, Ltd); these were cooled and measurements made in a temperature-controlled refrigerator. The mean diameter of the water spheres was found by microscopy to be $\sim 40\ \mu\text{m}$, but the spread is an order of magnitude in each direction. The Wayne-Kerr D121 permittivity cell and General Radio GR1621 precision measurements system were used. At each volume concentration, including 0% water with emulsifier present, three measurement runs were taken at 2°C intervals, and the mean value of the mixture permittivity ϵ_m taken.

According to the Maxwell-Lewin dielectric mixture formula⁴ for a volume fraction, ν , of spheres of substance 1 (water) in a matrix of substance 2 (*n*-hexane)

$$\frac{\epsilon_m - \epsilon_2}{3\epsilon_2} = \nu \left[\frac{\epsilon_1 - \epsilon_2}{2\epsilon_2 + \epsilon_1} \right]$$

Since in the temperature range 0 – 20°C the low frequency permittivity of water ϵ_1 is known accurately⁵, the formula can be fitted to the best value of ν , taking for ϵ_2 the present 0% data. The value of ν is found to be within 0.7% of the volumetrically-determined value over the 0 – 20°C range; there are a number of errors and effects contributing to this apparent volume defect, including the molar volume of the surfactant molecules. Assuming the value of ν calculated thus to be independent of temperature, ϵ_1 can be calculated from the data at temperatures $\leq 0^\circ\text{C}$. The consistency between the 1%

Fig. 1 The temperature dependence of the real part of the dielectric constant of water. $\circ$, 1% volume fraction emulsion; $\bullet$, 0.5% emulsion.

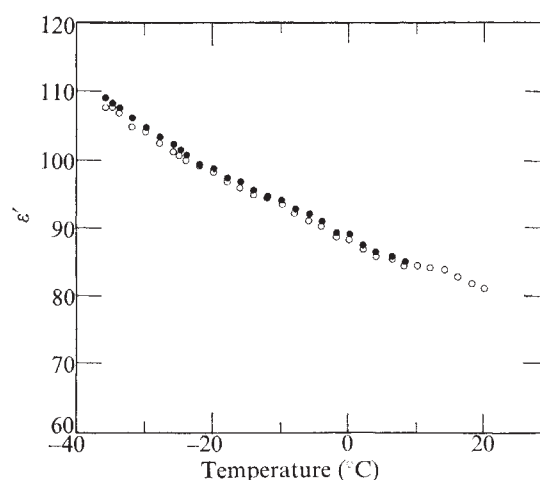


Table 1 Best values for temperature dependence of the permittivity of water

$T(^{\circ}\text{C})$	ϵ (water)
-35	107.7
-30	104.2
-25	100.8
-20	98.3
-15	95.6
-10	93.4
-5	90.9

and 0.5% data in Fig. 1 is such that $\pm 2\%$ confidence limits might be placed on the mean of the two sets. The neglect of the temperature variation of ν from thermal expansion gives rise to a positive error of 0.2% at -35°C , with correspondingly smaller errors at higher temperatures. Neglecting these errors, the best values are given in Table 1. They fall well outside the polynomial fit to the data that has been made at temperatures $\geq 0^{\circ}\text{C}$ (ref. 5), a feature of supercooling which has been noted in other polar liquids⁶.

There is apparently very little Maxwell-Wagner effect arising from conduction processes. A detailed study of the dielectric relaxation processes in the supercooled state is planned.

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Received June 10; accepted July 15, 1976.

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Conformation of Met⁵-enkephalin determined by high field PMR spectroscopy

THE discovery by Hughes *et al.*¹ that peptides (the enkephalins) could be the endogenous agonists of opiates in brain represents a challenge to medical chemistry both in its fundamental aspects and for the possible development of new therapeutic approaches. To understand how non-peptidic substances belonging to various chemical classes can mimic the action of the pentapeptide enkephalins it is first necessary to determine the spatial arrangement of the latter. We have thus synthesised large quantities of Met⁵-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and determined its preferential conformation in solution using high field proton magnetic resonance (PMR) spectroscopy at variable temperatures, coupling constants in relation with Karplus-Bystrov curves² and conformational energy steric maps³.

The most characteristic features of this conformation, which could account for some of the physicochemical and biological properties of the molecule seem to be: (1) a highly folded structure; (2) a precise homology between morphine and Met⁵-enkephalin as regards their respective functional groups (the phenol ring corresponding to the Tyr residue and the tertiary amine corresponding to the terminal ammonium function of the peptide); and (3) a relative freedom of the N-terminal peptide Tyr-Gly.

Met⁵-enkephalin was prepared by liquid phase synthesis (unpublished). Its 300-MHz PMR spectrum was recorded in DMSO_d₆ in which it dissolves readily (Fig. 1). All the ³J_{NH-CH_α} and ³J_{CH_α-CH_β} have been measured experimentally and the most probable values of torsional angles ϕ and ψ

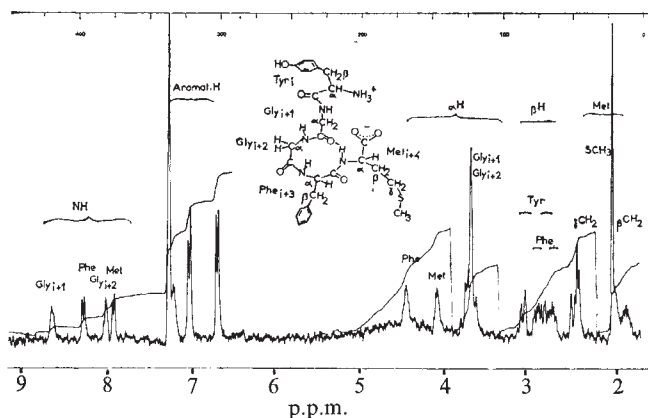


Fig. 1 300-MHz spectrum of Met⁵-enkephalin (5×10^{-1} M) in DMSO_d₆. Probe temperature, 17°C . TMS as internal reference.

were determined from the Karplus-Bystrov curves and conformational energy steric maps, respectively. The chemical shift variation of the Met NH proton, as a function of temperature, is somewhat lower than that of the other NH protons of the molecule, suggesting that this proton is involved in a hydrogen bond or is in a buried position. Only minor changes in coupling constants were observed with temperature variation, indicating that in the present conditions, the conformation is well defined, and corresponds to a relatively deep energy well. The conformational behaviour of Met⁵-enkephalin in aqueous medium also parallels very closely that found in DMSO (our unpublished results). On the basis of these results, we propose the conformation reported in Fig. 2 with a β_1 turn⁴ involving the sequence Gly-Gly-Phe-Met and a hydrogen bond between the NH of the Met_{i+4} and the CO of the Gly_{i+1} following the Tyr residue. In this conformation, the two aromatic residues (Tyr and Phe) are pseudoequatorial and the side chain of Met is pseudoaxial to the mean plane of the peptide backbone.

Interestingly, in this structure, the three hydrophobic chains (Tyr, Phe, Met) are situated at the periphery of the pseudocycle of the β_1 turn and the NH₃⁺ and the COO⁻ terminal groups exhibit a spatial proximity (end-to-tail interaction). These

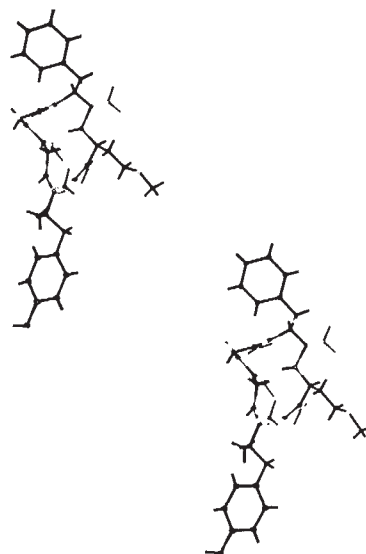


Fig. 2 Representation of the characteristic parts of opiates (left) and opiate-like peptides (right) exhibiting structural homology.

features probably account for the marked hydrophobic properties of Met⁵-enkephalin (water solubility < 0.7%), which suggest that the peptide could cross lipid-containing membranes with relative ease, including the so-called 'blood-brain barrier', a property which might be of considerable therapeutic interest.

The conformation of Met⁵-enkephalin deduced experimentally from its spectra in solution is different from the hypothetical conformation recently proposed⁶ and characterised by a β turn involving a hydrogen bond between the CO of the Tyr residue and the NH of the Phe residue. Nevertheless, the preferential conformation of Met⁵-enkephalin that we propose also shows all the steric requirements found in morphine for a stereospecific interaction with the receptor. In the N-terminal part of the molecule, the distance between the OH phenolic group and the ammonium function are the same, namely 6.8 Å, in morphine and in the proposed conformation of Met⁵-enkephalin (Fig. 3). Moreover, the spatial orientation of the ammonium groups in respect to the phenol rings is similar in both compounds.

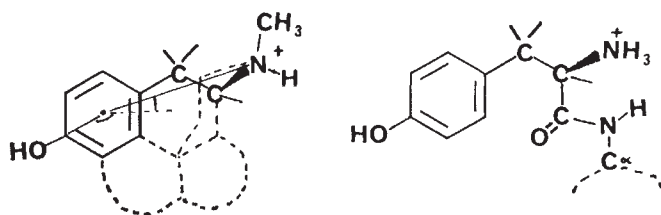


Fig. 3 Stereoscopic view of enkephalin along the mean plane of β_1 turn with $\psi_1=0^\circ$ (to allow for end-to-tail association), and $\phi_{1+4}=-160^\circ$ (avoiding steric strain for the side chain of methionine). Carbonyl peptide functions are visualised by bend bonds, C-N bonds are recognised by their change in colour (black-grey). The head (Tyr) is at bottom right, the tail (Met) at top right. The methionine side chain is pointing towards the observer (axial character); that of Phe has a pseudo-equatorial character. The aromatic rings are almost coplanar to the β_1 turn. Typically, NH_{1+4} is the only peptide NH proton pointing inwards.

The structural analogies between Met⁵-enkephalin and opiates suppose the existence of the opiates in the protonated form at physiological pH. Any modification of the N-terminal Tyr leading to a non-protonated form at this position should therefore inhibit biological activity. This assumption seems to be substantiated by the observation that substitution of the N-terminal function of the Tyr residue in opiate-like peptides leads to a loss of affinity for the receptor⁶.

In addition, it should be stressed that the β_1 turn gives a large degree of freedom to the Tyr-Gly moiety towards the rest of the molecule and this could be of importance in understanding the activity of other peptides with opiate-like activity. The primary attachment of Met⁵-enkephalin to the receptor could conceivably involve the Tyr-Gly moiety and the relative freedom of this moiety would allow the secondary attachment of the hydrophobic part of the molecule. It can be assumed that similar features (presence of an N-terminal Tyr-Gly moiety in a state of relative freedom) could account for the common affinity of many peptides like the two enkephalins, lipotropin C and the synthetic compounds of Goldstein *et al.*⁶. The relative differences in affinity between these compounds would depend essentially on the secondary interactions (for example, hydrophobic, hydrogen bond). The decreased affinity in Leu⁵-enkephalin compared with Met⁵-enkephalin¹ could thus be attributed to the loss of a hydrogen bond involving the sulphur atom of the Met residue.

It is hoped that these features of enkephalins will facilitate synthesis of opiate-like peptides of high potency and appropriate pharmacological properties, particularly in connection with the search for a non-addictive analgesic⁷.

This work was supported by the CNRS, the University Rene Descartes and the University of Gent. We thank Professor J. C. Schwartz, Unité de Neurobiologie, INSERM, Paris, for discussions and Dr J. M. Lecomte for assistance.

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Received April 30; accepted June 26, 1976.

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Proton magnetic resonance studies of conformation and flexibility of enkephalin peptides

ENKEPHALIN, an endogenous pentapeptide which has the analgesic properties of morphine¹, has recently been isolated, purified and characterised both chemically and pharmacologically^{2,3}. The fact that enkephalin and the opiates interact with the same receptor raises the possibility that the similarity in their function might be based on common structural features^{4,5}. Particular attention has been paid to the geometric relationship between the aromatic ring of the tyrosine residue and the terminal amino group of enkephalin compared with the known geometry of similar functional groups in morphine. The relative spatial disposition of the phenylalanine and tyrosine aromatic side chains is also of special interest because of the possible resemblance of enkephalin to oripavine^{4,5}. So far, however, no experimental determination of conformational parameters has appeared. Here we report proton magnetic resonance (PMR) studies of Met⁵-enkephalin which indicate that the methionine amino proton is involved in a hydrogen bond, most probably within a Gly-Gly-Phe-Met type I β turn, but not with a turn involving Tyr-Gly-Gly-Phe as proposed previously⁶. Although our data exclude a γ turn, they do not rigorously exclude other conformations, especially if these have small statistical weights.

Assignments of the 270-MHz PMR spectrum made by double resonance and difference double resonance are shown in Fig. 1. The only possible ambiguity in assignment, distinguishing Gly₂ and Gly₃, was removed by titration from ²H₆-DMSO into ²H₂O, the assignments in ²H₂O having been previously made by pH titration. The chemical shifts and scalar coupling constants obtained by spectral analysis are shown in Table 1. The side-chain rotamer populations derived from these are also listed.

It has been demonstrated^{7,8} that very small temperature dependencies such as observed here for Met (Fig. 2) are strong evidence for a hydrogen bond involving that amide proton. The coupling constants (³J_{NH-CH}) in Table 1 enable us to rule

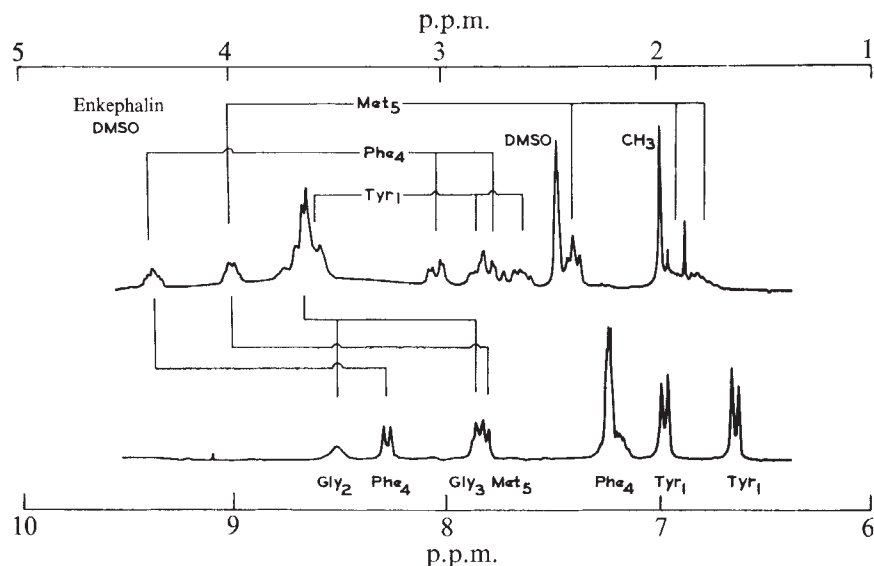


Fig. 1 PMR spectrum of Met⁵-enkephalin in ²H₆-DMSO. The Tyr₁-OH and NH₃ signals are not present and the Gly₂-NH is broadened. Scale is p.p.m. from H¹MS.

out the possibility that the hydrogen bond is involved in a γ turn⁹ and are instead indicative of a type I β turn. A type II β turn would not be in such good agreement with the observed coupling constants as a type I β turn but cannot be rigorously excluded by these data. Further studies of the Gly₂ and Gly₃ residues are needed since accidental degeneracy of the C α protons has so far prevented an analysis of the NH-CH₂ spin system of Gly₃, and since the two ³J_{NH-CH} values for Gly₂ are not available from the spectrum because of line broadening. A similar line broadening has been observed in oxytocins¹⁰.

conclusions can be drawn from this fact. First, there are $3^3 = 27$ possible forms of enkephalin which differ in the orientation of at least one of these three side chains. Second, for each of these three amino acids the Boltzmann energy differences (ΔE) between the various rotamer states must all be $\sim RT$. Consequently, we conclude that there may not be a simple direct relationship between the rotamer preferences observed here and the side-chain conformation on the receptor. Note, however, that a *trans-gauche* isomer of tyrosine (Table 1) is required to give a tyrosyl geometry resembling morphine.

Clearly, the more stable a conformational feature is, the more likely it is to be preserved in the substrate-receptor complex. Since β turns are often stabilised to the extent of several kcalories, the β turn which we are suggesting for the solution conformation of enkephalin may be an important feature in the interaction of enkephalin with its receptor. There are two pieces of indirect evidence which support this. First, a model of enkephalin in a type I β turn involving the Met-Phe-Gly-Gly residues shows a distinct topological resemblance to oripavine in the relative disposition of the tyrosyl and phenyl rings. The importance of the conformation of the β turn and Phe is clearly shown by the fact that the replacement of L-Phe by D-Phe in Met⁵-enkephalin results in a peptide which is inactive (Garsky *et al.*, unpublished). Although a β turn of the residues Tyr-Gly-Gly-Phe (ref. 6) also resembles oripavine, the chemical shift data in Fig. 2 show no evidence for a hydrogen bond between the amino group of Phe and the carbonyl group of tyrosine⁶. Second, the side-chain rotamer populations of Phe exhibit the normal tendency toward $a = b = c = 1/3$ as temperature is increased (Fig. 3). This behaviour is indicative of a situation in which there is only one degree of rotational freedom (that is, the rotation about the C α -C β bond in this case) which has Boltzmann energy differences $\Delta E \sim RT$ and is the behaviour we find in other peptides with rigid ($\Delta E > RT$) backbone structures. In contrast, non-rigid backbones ($\Delta E \sim RT$) would be expected to add considerable complexity to the temperature dependence of side-chain rotamer populations. As Fig. 3 shows, both the Met and Tyr side-chain rotamer populations show a less conventional temperature dependence than Phe and this may indicate greater flexibility for the ϕ , ψ angles of Met and Tyr than for the corresponding angles involved in the β turn.

Our data are consistent with a backbone conformation of enkephalin having a type I β turn with a hydrogen bond between the amide proton of Met₅ and Gly₂ (Fig. 4). Correlation of this conformation and that of morphine with their respective biological functions is not simple. The predominant *trans-gauche* geometry of the Tyr₁ residue certainly correlates

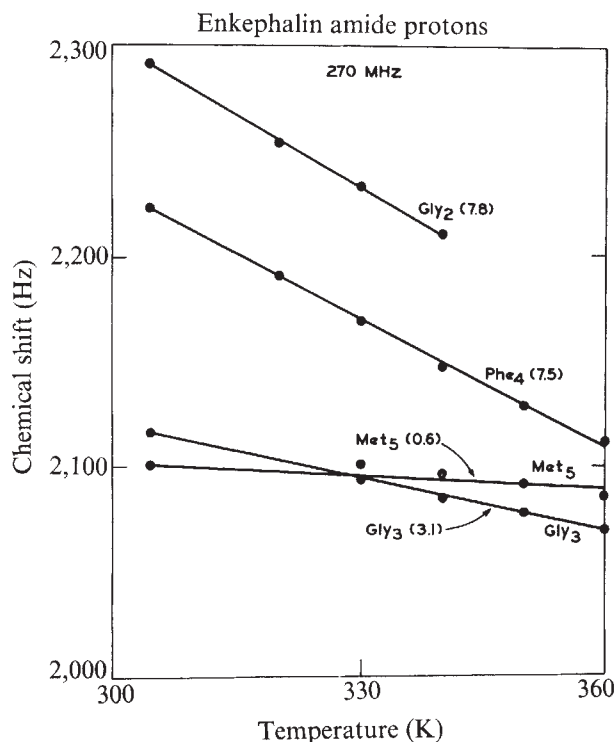


Fig. 2 Effect of temperature on the amide proton chemical shifts. The slopes (p.p.10³ per °C) are given in parentheses.

The analysis of the side-chain rotamer population¹¹ (Table 1) shows that the Met, Phe, and Tyr side chains favour one of the two possible *trans-gauche* rotamers¹², but for each of these residues all three rotamers have significant populations. Two

Fig. 3 Effect of temperature on the rotamer populations of Met⁵-enkephalin (calculated by the method of Pachler¹¹). The third rotamer population can be obtained from the graphs, since $a + b + c = 1$.

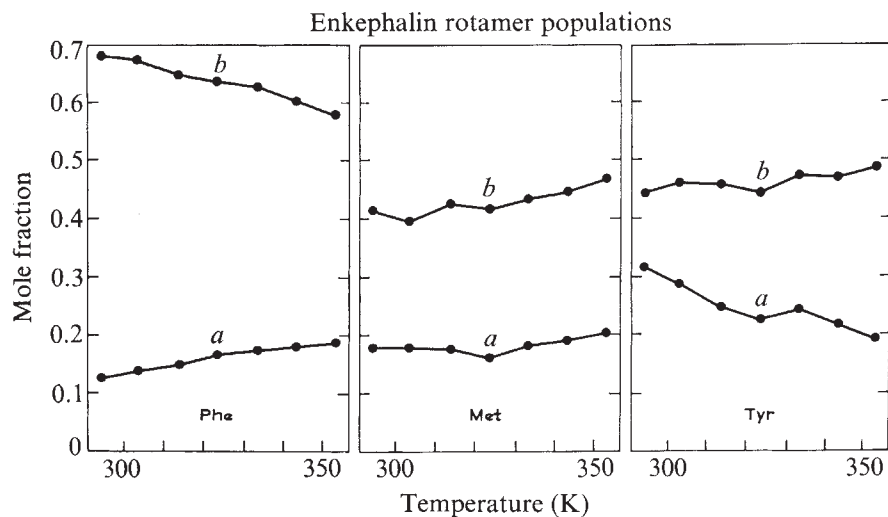


Table 1 PMR parameters for enkephalin in ²H₆-DMSO (2 mg per 0.5 ml) at 25 °C

Chemical shift*	Tyr ₁	Gly ₂	Gly ₃	Phe ₄	Met ₅
δ CαH	3.61	3.67	3.74	4.35	3.96
δ CβH	2.85	—	—	3.04	1.92
δ CβH	2.67	—	—	2.78	1.79
Coupling constant†					
³ J α β1	6.2	—	—	4.0	4.5
³ J α β2	7.5	—	—	10.1	7.3
² J α ββ	-13.7	—	—	-13.9	-13.3
³ J _{NH-CH}	—	—	—	8.3	7.2
Rotamer populations‡					
g ⁺ g ⁻	0.33	—	—	0.13	0.17
tg ⁺	0.44	—	—	0.68	0.43
tg ⁻	0.23	—	—	0.19	0.40
Temperature dependence of amide shifts δ	—	-7.8	-3.1	-7.5	-0.6

* Units = p.p.m. from hexamethyldisilane (HMS).

† Hz at 270 MHz.

‡ Statistical weights of rotamers calculated according to Pachler¹¹ parameters. This method does not unequivocally distinguish the tg⁺ from tg⁻ rotamers.

§ Units = p.p.10⁹ per °C.

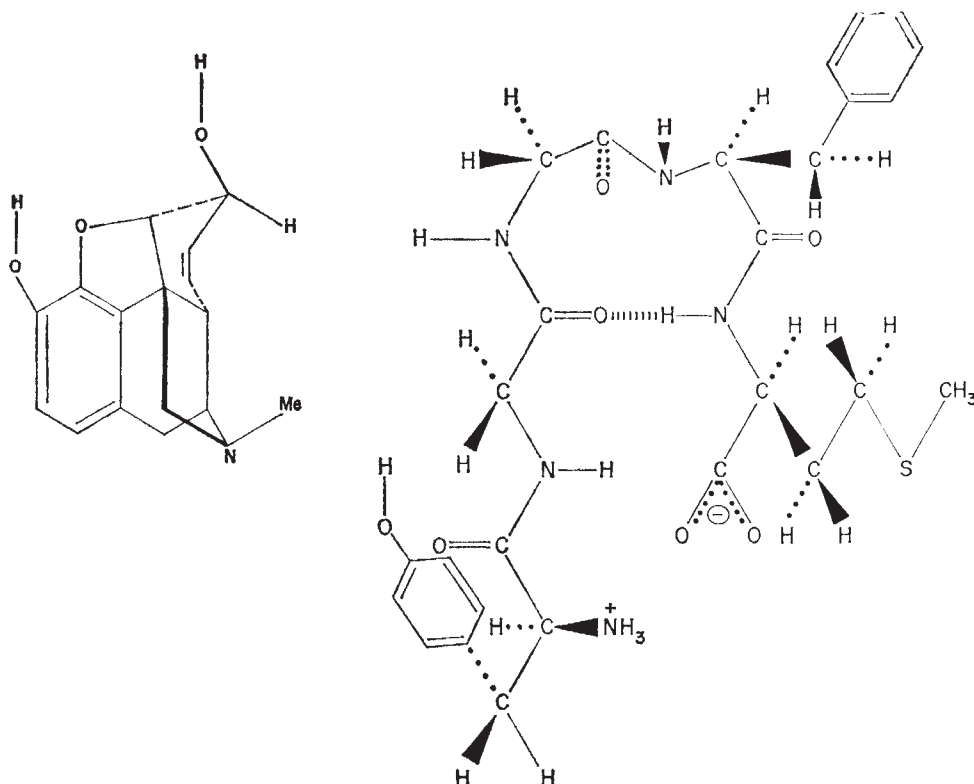


Fig. 4 Diagrammatic representation of one conformation of Met⁵-enkephalin. Note the β turn, the proximity of the NH₃⁺ and CO₂⁻ groups, the conformation of the Tyr₁ residue, and the L-Phe in the β turn.

with the known steric requirements of morphine^{4,13}, but there is not yet a simple explanation of the requirement for a type I β turn (except that L-Phe and not D-Phe is active) or that there can exist 27 rapidly interconverting enkephalin forms. The time scale for this rearrangement will be approximately 10^{-10} – 10^{-11} s, which is faster than the residence time of the hormone-receptor complex. Consequently, all 27 enkephalin forms may bind initially to the receptor which then selects the appropriate form in accordance with the induced-fit theory of hormone action¹⁴.

In H₂O solution, both Gly₂ and Gly₃ NH-CH₂ spin systems are degenerate as ²H₆-DMSO and the Phe₄ and Met₅ have ³J_{NH-CH} values close (1 Hz) to the value in ²H₆-DMSO. The interpretation of these data is that the enkephalin conformations and internal motions are qualitatively similar in both solvents. Leu⁵-enkephalin has basically the same conformation as Met⁵-enkephalin in ²H₆-DMSO.

This research was supported by the Colleges of Agriculture and Life Sciences of the University of Wisconsin-Madison, and by the NIH and the NSF. C. R. J. was supported by an Enzymology Postdoctoral Training Grant.

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Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates

SEVERAL groups of workers have recently demonstrated the existence of naturally-occurring peptides which show agonist activity at the opiate receptor^{1–4}, and Hughes *et al.*⁵ have shown that the opiate activity isolated by them is due to a mixture of two peptides H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH termed methionine-enkephalin and leucine-enkephalin respectively.

The agonist activity of these two peptides was established using *in vitro* techniques and early work² demonstrated that the enkephalins were subject to rapid deactivation when exposed to tissue homogenates and purified enzyme preparations such as carboxypeptidase-A and leucine aminopeptidase. In view of these results, it was perhaps not surprising to find that *in vivo*, even after central administration, the antinociceptive actions of the peptides are, at best, transient (refs 6 and 7 and G. Metcalf, personal communication). Using the depressant effects of the enkephalins on the electrically-evoked contractions of the mouse vas deferens as a bioassay, the half-life of both peptides in rat plasma was shown to be very short (Table 1). In this study we attempted to identify the mode of

deactivation of the peptides in rat plasma and brain homogenates, which we believe could be responsible for the limited *in vivo* activity of the enkephalins.

³H-Gly³-Met⁵-enkephalin was synthesised from ³H-glycine using solutions methods¹⁵ 3,5-³H-Tyr-Leu⁵-enkephalin was synthesised from the unlabelled pentapeptide by the Radiochemical Centre, Amersham.

Met⁵-enkephalin, Leu⁵-enkephalin, H-Tyr-Gly-Gly-Phe-OH, H-Gly-Gly-Phe-Met-OH, and H-Gly-Gly-Phe-Leu-OH were synthesised by standard methods¹⁵. Thin-layer chromatography was carried out on silica plates (Kieselgel F₂₅₄, Merck Ag) using the following solvent systems: (1) chloroform-methanol-acetic acid-water, 45:30:6:9 (2) *n*-butanol-pyridine-acetic acid-water, 60:20:6:24 (3) *n*-butanol-ethyl acetate-acetic acid-water, 1:2:1:1 (4) chloroform-methanol-acetic acid, 18:2:1. Distribution of radioactivity on the plates was determined using a zonal scraping technique. Column chromatography was carried out on DEAE-Sephadex in the acetate form with 1% pyridine, 0.04% acetic acid as eluting solvent. Peptides were hydrolysed in 6 M hydrochloric acid in sealed tubes (108 °C; 20 h) before analysis using a Jeol 6 AH automatic amino acid analyser.

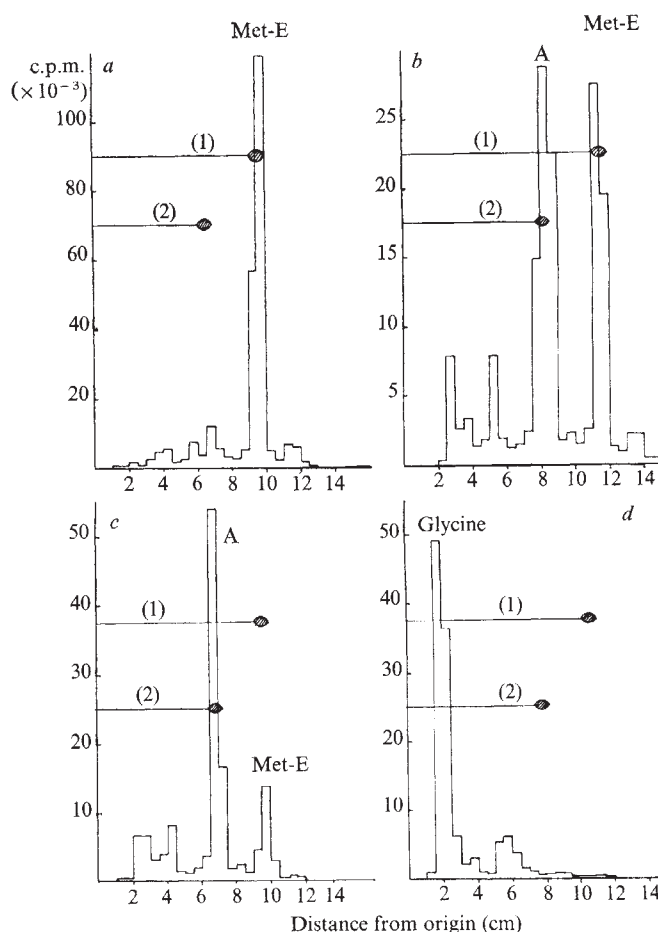


Fig. 1 Breakdown pattern of ³H-Gly³-Met⁵-enkephalin (0.3×10^{-6} M) at 37 °C by rat plasma (20% v/v) in 50 mM Tris-HCl buffer (pH 7.4). a, 0 min; b, 5 min; c, 10 min; d, 30 min. Samples (0.2 ml) were taken periodically, added to methanol (0.1 ml) and stored in ice. The supernatants were applied to silica plates and eluted with solvent system 1. Authentic standards were visualised with ninhydrin, the plates divided into bands (0.5 cm) and the silica scraped into scintillation vials. Water (2.0 ml) and an aliquot (5 ml) of a solution of butyl-PBD (7.5 g) in toluene (666 ml) and Triton X-100 (333 ml) were added and the vials shaken to form a stiff gel. Radioactivity was determined using a Packard 2450 scintillation spectrometer. The histograms represent the distribution of radioactivity (c.p.m.) on the plates with reference to the *R_f* values of authentic standards: 1, Met⁵-enkephalin; 2, H-Gly-Gly-Phe-Met-OH.

Initial studies were carried out at 37 °C with ^3H -Gly 3 -Met 5 -enkephalin (0.3×10^{-6} M) in an incubation mixture consisting of rat plasma (20% v/v) in 50 mM Tris-HCl (pH 7.4). These showed a breakdown pattern for the pentapeptide which is represented by the histograms in Fig. 1. At this concentration, the disappearance of Met 5 -enkephalin was rapid, which is consistent with the rapid deactivation of the peptide in rat plasma. Continuation of the incubation resulted in the recovery of essentially all radioactivity as ^3H -glycine, indicating complete degradation of the peptide under these conditions. The main product (A), which was somewhat more stable than the parent pentapeptide, appeared to be chromatographically similar to the tetrapeptide H-Gly-Gly-Phe-Met-OH (all possible Gly 3 -containing peptides were used as chromatographic standards). Product A was isolated from a larger scale incubation by chromatography on DEAE-Sephadex and purified by preparative thin layer chromatography on silica plates. After acid hydrolysis, the amino acid composition was determined and found to be compatible with the structure H-Gly-Gly-Phe-Met-OH (glycine 1.83, phenylalanine 1.17, methionine 0.85); no tyrosine was detected. The metabolite proved to be chromatographically identical to the synthetic tetrapeptide on silica plates using the solvent systems above. On the basis of this evidence, the primary step in the breakdown of Met 5 -enkephalin in rat plasma is assigned as cleavage of the Tyr-Gly amide bond. As the tetrapeptide H-Gly-Gly-Phe-Met-OH shows very little activity using the mouse vas deferens assay procedure (Table 1), this cleavage is consistent with the deactivation of Met 5 -enkephalin in rat plasma.

Table 1 The depressant effects of the enkephalins and degradation products on the electrically induced contractions of the mouse vas deferens

Structure	Relative activity* mouse vas deferens	t_1 (rat plasma)† (min)
H-Tyr-Gly-Gly-Phe-Met-OH	100	2.0
H-Tyr-Gly-Gly-Phe-Leu-OH	170	2.5
H-Gly-Gly-Phe-Met-OH	0.1	—
H-Gly-Gly-Phe-Leu-OH	0.1	—

*Determined by the method of Henderson *et al.*⁸.

†Rat plasma containing the test compound ($100 \mu\text{g ml}^{-1}$) was incubated at 37 °C. Samples (0.1 ml) were taken periodically and added to 0.01 M hydrochloric acid (0.1 ml). Each sample was diluted ($\times 10$) with Krebs solution and aliquots (0.1–0.3 ml) of the resulting solution injected into an organ bath (4 ml) containing the vas deferens. The depression in twitch height obtained with each sample was compared to a dose-response curve constructed for authentic methionine-enkephalin and the half life of biological activity determined.

Experiments carried out using human plasma showed that an essentially identical pattern of breakdown was taking place, though the rate of cleavage seemed to be somewhat slower.

Incubation of ^3H -Tyr-Leu 5 -enkephalin with rat and human plasma in similar conditions resulted in the formation of a single radiolabelled product which was stable to further incubation. The R_f value of this product corresponded closely with that of tyrosine in the above thin-layer chromatographic systems, and when the product was applied to an amino acid analyser the radioactivity was eluted concurrently with authentic tyrosine. At no time was any radioactive product present which had chromatographic properties similar to any of the possible tyrosine-containing degradation products, authentic samples of which were used as chromatographic standards. This is consistent with ^3H -tyrosine being the primary labelled product of the degradation of ^3H -Tyr-Leu 5 -enkephalin. Thus it seems that Leu 5 -enkephalin is degraded in rat and human plasma in an identical way to Met 5 -enkephalin, that is, by cleavage of the Tyr-Gly amide bond.

Breakdown of the enkephalins takes place very rapidly in the presence of rat brain homogenates: ^3H -Tyr-Leu 5 -enkephalin (10^{-7} M) was completely destroyed within 1 min at 37 °C in a homogenate of whole rat brain containing approximately 3 mg protein ml $^{-1}$. The mode of deactivation seemed to be identical

to that observed in plasma for both enkephalins. The sub-cellular localisation of the enzyme(s) responsible for the cleavage of the Tyr-Gly bond is under investigation.

The observation that the enkephalins are subject to rapid deactivation mediated by cleavage of the Tyr-Gly bond in both plasma and brain is compatible with the observed properties of the peptides *in vivo*^{6,7}. It has been reported that the addition of bacitracin (a polypeptide antibiotic which has been shown to be a potent glucagon inactivation inhibitor¹⁰) enhances enkephalin-mediated effects on cyclic GMP accumulation in slices of rat neostriatum⁹. This enhancement may well be due to inhibition of peptidase activity by the antibiotic. It is interesting to note that, despite the lability of the peptides in brain homogenates, several groups of workers have been able to demonstrate the affinity of the enkephalins or related peptides for the opiate receptor in brain^{3,4,11,12}.

The important observation^{7,12} that porcine β -lipotropin C-fragment (a 31-residue peptide isolated from pig pituitary which has the Met 5 -enkephalin sequence at its N terminus¹³) shows potent and long lasting morphine-like effects in the cat is surprising in view of its structural relationship to the enkephalins. It is possible, however, that the tertiary structure of the longer peptide is such that the labile Tyr-Gly bond is protected from the metabolising enzyme, thus conferring greater stability on the molecule *in vivo*.

In conclusion, the deactivation of the enkephalins in rat and human plasma and in rat whole brain homogenate has been shown to take place through cleavage of the Tyr-Gly amide bond. The localisation, nature and specificity of the enzyme system responsible for the cleavage is unknown but its precise role and mode of action may be expected to have a major effect on the pharmacology and biochemistry of the enkephalins and related compounds.

Since this paper was submitted, further evidence that cleavage of the Tyr-Gly amide bond is the initial deactivation step of the enkephalins *in vivo* has been provided by structure-activity studies on enkephalin analogues. In particular D-Ala 2 -Met 5 -enkephalin is extremely resistant to plasma enzymes. This superactive analogue ($5 \times$ Met 5 -enkephalin potency in the mouse vas deferens assay) has a t_1 of at least $20 \times$ Met 5 -enkephalin in rat plasma indicating the importance of the Tyr-Gly region of the molecule to the substrate specificity of the degrading enzyme(s). Similar results have been reported by other workers¹⁴.

We wish to thank Dr J. D. Bower, Mrs C. Calverley, Mr P. Coffin, Mr R. Pape, Mr J. D. Robinson and Mr A. Wilson for their technical assistance.

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Received June 7; accepted July 15, 1976.

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Substance P and analgesia

SUBSTANCE P (SP)¹ has long been known to have marked effects on the central nervous system (CNS). Lembeck² suggested that SP might be a transmitter of primary sensory impulses, an hypothesis supported by subsequent investigators^{3,4}. Early work on SP was carried out using natural material, which unless highly purified, is known to be contaminated with bradykin or some other kinin-like material⁵. As bradykin also has marked effects on the CNS, experiments carried out using impure SP may be misleading. This may account for some of the conflicting observations that exist regarding the action of SP on sensory transmission, and may also explain its interaction with opioids. Whereas impure SP has been shown to antagonise morphine⁶, synthetic SP was found to substitute effectively for morphine in mice chronically treated with morphine⁷. SP does not act like morphine in the field-stimulated guinea pig ileum⁸ and does not combine with morphine receptors⁹. Furthermore, SP has been reported to be the most potent algogenic substance known on the human blister base¹⁰. Recent reports on the characterisation and activity of enkephalin^{11,12} suggested that enkephalin may be acting as an analogue of SP. We have therefore examined SP for morphine-like activity.

This hypothesis was tested initially by examination of the effects of intracerebral injection¹³ of SP and morphine into 30-g male Swiss Webster mice. Response of the animals to heat stimuli was tested using a hot plate analgesimeter¹⁴. Each mouse's reaction time (RT; s) was determined before administration of any drug and again at 30-min intervals after drug injection. Naloxone and morphine were administered in physiological saline; SP was administered in physiological saline containing 0.01 N acetic acid, to prevent adsorption of SP to glass. When used, naloxone was injected intraperitoneally immediately before intracerebral injection of other drugs. Vehicle controls were carried out. The pre-drug RT was subtracted from each of the RTs observed after treatment, and these differences in RT are reported in Table 1.

SP and morphine each caused an increase in RT, indicating analgesia; and the analgesic action of each was prevented by naloxone. Mice which received SP seemed to be quiet compared with normal mice. They would cease movement, seeming to sleep, often in an abnormal posture. These animals were readily aroused, and seemed to be alert,

Table 1 Analgesic action of intracerebrally administered SP and morphine sulphate (MS)

Treatment	Increase in RT at:		
	30 min	60 min	90 min
SP (7)	3.0±0.83	5.23±0.72	8.23±1.09
SP vehicle (7)	-0.11±1.57	1.43±1.28	2.03±1.53
MS (7)	8.03±1.75	4.86±1.46	2.49±0.61
Saline (7)	-1.14±0.51	-0.41±1.16	0.63±0.6
SP+naloxone i.p. (7)	-0.54±0.23	1.11±0.48	2.97±0.52
SP+SP vehicle i.p. (7)	3.6±1.32	4.77±1.85	7.23±1.44
MS+naloxone i.p. (5)	0.0±0.93	1.84±1.15	4.52±1.92
MS+saline i.p. (5)	8.69±4.50	7.32±3.97*	5.80±2.76*
Naloxone i.p.+SP vehicle i.c. (7)	-0.74±1.06*	-0.31±0.67*	
Saline i.p.+SP vehicle (7)	-0.23±0.84*	-0.31±0.88*	
Chronic MS i.p.+SP (7)	0.37±0.92	-0.26±0.72	1.51±0.66
Chronic MS i.p.+MS (5)	-0.92±1.41	1.72±1.39	0.32±1.48
Chronic saline i.p.+SP (5)	3.77±1.70	7.50±2.11	7.90±1.83

Injections were in 0.04 ml intracerebrally (i.c.) and 0.1 ml intraperitoneally (i.p.). Doses (per mouse): SP 2 ng; MS 0.4 µg; naloxone 60 ng (i.p.). All increases in RT were statistically significant with respect to the corresponding controls using Student's *t* test ($P<0.05$) except those marked with an asterisk.

Number of animals per group is given in parentheses. All drug injections were made at 1030.

Table 2 Analgesic action of intraperitoneally administered SP

Time of testing (min)	Increase in RT (s)		
	Group 1	Group 2	Group 3
30	7.04±1.91	7.6±2.69	0.3±1.48
60	6.56±2.4	11.44±3.49	2.08±1.48
90	8.36±3.2	10.96±1.33	1.12±1.41
120	6.24±1.4	8.2±3.18	3.0±0.86
150	3.84±2.12	4.08±1.26	2.32±1.20

Each group contained 5 mice, each weighing 30 g. Injections (0.1 ml intraperitoneally) were: group 1, SP 5 ng per mouse; group 2, SP 1 µg per mouse; group 3, SP vehicle alone. For other details of testing, see text and Table 1. Increased RTs of animals in groups 1 and 2 were all significant compared with group 3 ($P<0.05$) except at 150 min.

however, while being handled and while on the analgesimeter. These behavioural effects of SP were similar to those described previously¹⁵.

To determine whether animals made tolerant to morphine also showed cross tolerance to the analgesic action of SP, animals were given morphine chronically before testing. On the first day of this experiment the RT of each mouse was tested on the analgesimeter before drug or placebo administration. Mice were then divided into three groups. Groups 1 and 2 received three injections of morphine intraperitoneally (0.25 mg per mouse per injection) on day 1, three injections on day 2 (0.5 mg) and three injections on day 3 (1.0 mg). On day 4 mice of group 1 were given SP intracerebrally and the mice of group 2 were given morphine intracerebrally. Animals of group 3 were given saline injections on days 1, 2, and 3, and received SP on day 4. The last part of Table 1 shows that mice treated chronically with morphine did not show any analgesic reaction to morphine or SP, whereas mice given chronic saline responded normally to SP.

Since SP has been reported to cross the blood-brain barrier⁷, it was also tested for analgesic action after peripheral administration. The experimental protocol was similar to that of the initial experiment described above, except that SP was administered by intraperitoneal injection. Table 2 shows that SP caused significant analgesia in mice at dose levels of 5 ng or 1 µg per mouse. At all times except 150 min, the changes in RT of animals in groups 1 and 2 were statistically significant compared with vehicle controls (group 3). The changes in RT at 150 min were not statistically significant, probably because of the small number of animals used and the fact that the analgesia was wearing off at that time.

In view of these results, the reported algogenic action of SP on the human blister base was re-examined using synthetic SP. Blisters were raised on the volar surface of human forearms by application of Cantharone (0.7% cantharidin in collodion). After 20 h the blister top was removed and the fibrin layer removed from the base. Substances to be tested were dissolved in buffered physiological saline and applied to the blister base¹⁰. Synthetic SP was not algogenic at concentrations up to 1 mg ml⁻¹, whereas acetylcholine and bradykinin were potent algogens. Algogenicity of acetylcholine and bradykinin was not antagonised by SP or Leu⁵-enkephalin. The previously-reported¹⁰ algogenicity of SP on the blister base may have been attributable to kinin contamination, since the SP used was isolated material. The failure of SP to produce analgesia on the blister base suggests that its site of action is central, as is that of morphine. The interaction we observed between SP, morphine and naloxone also supports this postulate.

We have found SP to be a potent analgesic in mice. This action of SP *in vivo* is in sharp contrast to the reported failure of SP to act like morphine on the field-stimulated guinea pig ileum⁸ or in binding to morphine receptors⁹. Since SP is a powerful stimulant for contraction of the

ileum, it is possible that this latter activity may have obscured any morphine-like inhibition of contraction. With respect to receptor binding, it is possible that more than one type of morphine receptor exists in CNS tissue and that the one tested by Terenius⁸ may not combine with SP. The SP receptor may possibly be degraded during the isolation procedure. It is possible that the analgesic action of SP may be mediated through the release of other substances. Another possibility is that the analgesia after administration of SP is not attributable to the intact molecule, but a metabolite of it. The slow onset and prolonged duration of action of SP compared with morphine may lend support to this hypothesis. Arguing against this possibility is the observation of Otsuka *et al.*,⁴ who found that shorter C-terminal fragments of SP, which retain relatively high myotropic potency, had little depolarising activity in frog spinal motoneurons.

It has been reported that enkephalin, which comprises residues 61–65 of β lipotropin, has only very transient and probably insignificant analgesic action *in vivo*¹⁶, whereas the much larger C-fragment (residues 61–91 of β lipotropin) is an active, potent, *in vivo* analgesic¹⁷; these data were obtained by intracerebral injection of the peptides. Our finding of the potent analgesic action of SP after peripheral administration suggests that the development of a small peptide as a practical analgesic drug may be possible. The intraperitoneal route of administration should minimise any undesirable hypotensive effects of SP, and its already prolonged analgesic action may be further prolonged by depot forms of administration.

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Received June 14; accepted July 6, 1976.

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Colchicine inhibits mitogenesis in C1300 neuroblastoma cells that have been arrested in G₀

CELLS in G₁ or G₀ usually progress through the cell cycle and stop in metaphase when colchicine is added to the growth medium^{1–6}. I have observed, however, using two different experimental techniques, that mouse C1300 neuroblastoma cells reversibly arrested in G₀ by serum limitation⁷ are unable to re-enter the cell cycle in the presence of colchicine.

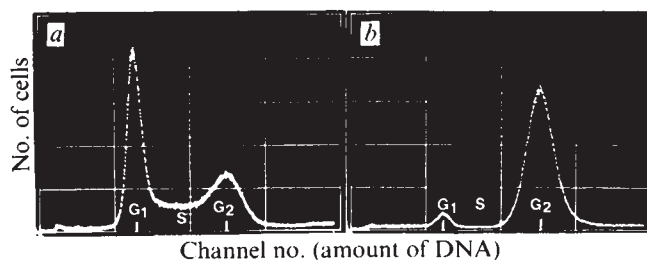


Fig. 1 Effect of colchicine on exponentially growing C1300 neuroblastoma cells. FMF analysis (a) before colchicine and (b) 40 h after addition of $1 \mu\text{g ml}^{-1}$ colchicine to the growth medium. C1300 cells were treated as described by Crissman and Tobey¹¹ to obtain a monodisperse suspension. Cells were stained with mithramycin and passed through a Los Alamos design microfluorometer¹⁰ equipped with an argon laser excited at 457 nm. The fluorescent light flash from each cell, which is proportional to the cell's DNA content, was converted to an electrical pulse by a photomultiplier and stored in a multichannel pulse-height analyser. The data were also displayed on an oscilloscope as shown above.

Colchicine inhibited by about 85% the incorporation of ³H-thymidine into DNA from 21 to 28 h after the arrested C1300 cells were returned to complete growth medium (Table 1). This could mean that C1300 cells do not leave G₀, or progress more slowly through the cell cycle, or do not take up thymidine⁸. A second technique, flow microfluorometric (FMF) analysis^{9–11}, which can determine the distribution in the cell cycle of a cell population by measuring the DNA content of individual cells, was used to distinguish among these possibilities. FMF analysis showed that after 44 h in growth medium plus colchicine about 85% of the original cell number is still in G₀ (Table 1). Colchicine therefore prevents C1300 cells from leaving G₀. The effect of colchicine additions at 10, 17, and 20 h after the cells were returned to DEM+10% calf serum (Table 2) suggests that the block involves the latter part of G₀ but not S phase. Colchicine inhibits mitogenesis in C1300 cells in G₀ but not

Table 1 Inhibition by colchicine of re-entry of C1300 neuroblastoma cells from G₀ into the cell cycle

Colchicine ($\mu\text{g ml}^{-1}$)	% Inhibition of TCA-precipitable ³ H-thymidine counts*	% Inhibition of release from G ₀ as determined by FMF analysis†
1	88	85
0.5	86	82
0.10	87	82

*C1300 cells, which were arrested in G₀ by serum limitation⁷, were plated in Dulbecco's modified Eagle's medium (DEM)+10% calf serum at 2.5×10^5 cells per 60-mm Petri dish (Falcon). Colchicine was added about 30 min later. Cultures were pulsed with $5 \mu\text{Ci } ^3\text{H}$ -thymidine in 0.10 ml $1.5 \times 10^{-4}\text{M}$ non-radioactive thymidine from 21 to 28 h after plating. Cells were collected on glass-fibre filter papers, washed twice with Tris-saline buffer, twice with 10% TCA and once with 95% ethanol. The filter papers were counted in a scintillation fluid containing 4.2% Liquifluor in a liquid scintillation counter. Each sample point was done in duplicate. The variation between points was less than 10%. These results are from one of several experiments, all of which showed this effect. The sample without colchicine added had 70,000 c.p.m.

Cells were monitored for their ability to maintain a healthy clumped morphology in the suspension culture and to exclude Trypan blue. At all colchicine concentrations, for the first 30 h of the experiment, the cells grew in healthy clumps in suspension and over 90% excluded Trypan blue. By 48 h, however cultures with $1 \mu\text{g ml}^{-1}$ colchicine had smaller clumps and only 75% of the population excluded Trypan blue. The cells appeared to deteriorate after 45 to 50 h at this colchicine concentration. At the lower colchicine concentrations, the cells appeared clumped and at least 85% excluded Trypan blue after 48 h.

†FMF analysis in parallel cultures to the first part of the experiment was carried out as described previously^{7,9–11} using the mithramycin staining procedure¹¹. FMF experiments carried out parallel with other ³H-thymidine incorporation experiments gave similar results.

Table 2 Interval of sensitivity to colchicine additions to cell cultures after arrested C1300 cells were returned to complete growth medium

Time of colchicine addition* (h)	% Inhibition of TCA-precipitable ³ H-thymidine counts†
1	88
10	79
17	31
20	15

*Results for colchicine concentration of $1 \mu\text{g ml}^{-1}$. Similar results were observed at colchicine concentrations of 0.5 and $0.1 \mu\text{g ml}^{-1}$.

†TCA-precipitable ³H-thymidine incorporation was measured 21 to 28 h after the arrested cells were returned to DEM+10% calf serum. Assay was carried out as described in Table 1 and ref. 7. In addition, FMF analysis was carried out on parallel cultures 48 h after the cells were returned to DEM+10% calf serum.

in G_1 , since only 5% of an exponentially-growing cell population is in G_1 (Fig. 1) 40 h after addition of $1 \mu\text{g ml}^{-1}$ colchicine to the growth medium. Vinblastine sulphate ($1 \mu\text{g ml}^{-1}$) also inhibits the re-entry into the cell cycle of C1300 cells arrested in G_0 .

The mechanisms of growth regulation in normal and transformed cells are still being elucidated^{6,7,12,13}. The cell membrane is thought to have a central role in controlling cell growth, in part by mediating the effects of serum factors that signal a cell to proliferate early in G_1 or G_0 (refs 14–17). This concept and the evidence that colchicine and other microtubule-disrupting drugs can affect the topographical distribution of receptors in the cell membrane^{18–23} could explain my results. Colchicine may be altering the topographical distribution of receptor(s) for serum factor(s) required by the arrested C1300 cells to re-enter the cell cycle.

The results presented in Table 2 show that C1300 cells can receive a 'stop' signal late in G_0 , some considerable time after the initial 'go' signal is received from serum factors¹⁷. This suggests that the mechanism of mitogenesis depends on specific receptor configuration(s) in late G_0 as well as in early G_1 or G_0 (refs 15–17).

Combinations of drugs that include a microtubule-disrupting agent are more effective than single drugs in treating tumours^{24,25}. The results presented here suggest that this may be due, in part, to the effect the microtubule disrupting agent has on cells in G_0 . The development of selective approaches to alter the mobility of receptors on the plasma membrane may prove useful in controlling malignant cell growth.

Gunther *et al.*²⁶ have shown that colchicine inhibits mitogenesis in lymphocytes. Also, colchicine inhibits mitogenesis in mouse 3T3 cells and dog MDCK kidney cells that have been reversibly arrested in G_0 (M. Baker, unpublished).

I thank Drs H. Friedman, R. W. Holley, J. Lockwood, G. Sato, J. Schneider, S. Varon, and the Human Biochemical Genetics Program, USPHS, for facilities and/or supplies. During part of this study, I was supported by a Senior Dernham Fellowship from the California Division of the American Cancer Society. I also thank Dr R. W. Holley for additional financial support (USPHS grant from the NCI) and encouragement; G. Baker, P. Schwalbe, and M. F. Weissberg for financial assistance; and Nathan Belcher, Pfizer Inc., for mithramycin.

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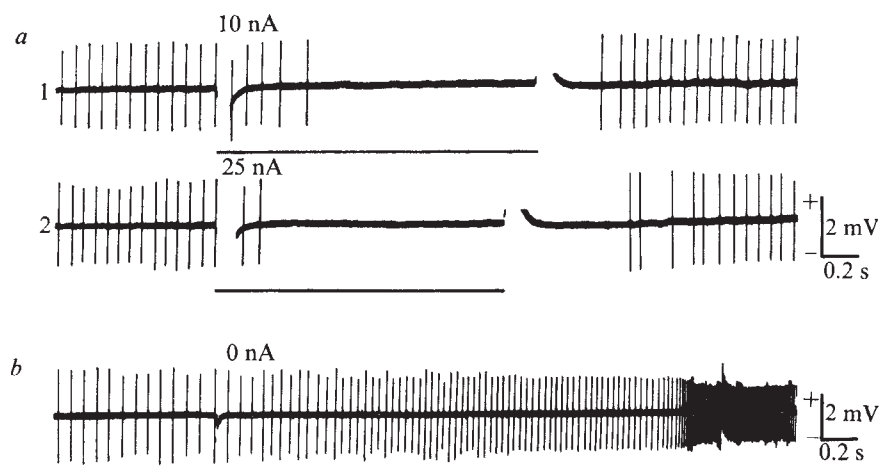
Inhibitory action of glutamic acid on cerebellar interneurons

GLUTAMIC acid strongly excites almost all central neurones examined, and it has been considered to be a main excitatory transmitter in the mammalian brain^{1,2}. Although some neurones in the olfactory bulb have been inhibited by glutamic acid³, this has been explained by the assumption that glutamate primarily activated γ -aminobutyrate (GABA)-releasing inhibitory interneurons, which in turn inhibited the neurones in question¹. In the experiments reported here, glutamate inhibited neurones in the granular layer of the cerebellum: this action does not seem to be mediated by inhibitory interneurons.

Thin sections of guinea pig cerebellum (80–120 μm thick) were prepared from the uvula and nodulus perpendicular to the long axis of the folium as before⁴. They were incubated in glucose-saline medium saturated with 95% O_2 and 5% CO_2 at 37 °C for more than 40 min, and transferred one by one into an observation chamber under a microscope⁵. Single cell discharges were recorded extracellularly from neurones in the granular layer with glass micro-electrodes of tip diameter 2–4 μm .

About half the neurones encountered in the granular layer were excited by glutamate (GE cells) and others were inhibited (GI cells) (Fig. 1). When glutamate was administered electrophoretically to a point about 30 μm from the tip of the recording pipette, the discharge rate of a GE cell started to increase within 1 s (Fig. 1b). The rate of firing of a GI cell, on the other hand, gradually decreased and then stopped (Fig. 1a, 1). Firing resumed after the electrophoretic current was turned off. An increase in electrophoretic current shortened the latency of inhibition (Fig. 1a, 2). In some GI cells, contact of a pipette containing glutamate with the section near the recording site induced suppression of the firing even if a retaining current of 25 nA was passed continuously through the pipette (Fig. 2a, 1). This was probably due to a background leakage of glutamate which was reported to occur occasionally in spite of the retaining current⁶. In these cells, reduction of the retaining current caused stronger inhibition, and reversal of the current further increased the inhibition. Therefore, the inhibition observed during administration of glutamate cannot be explained by the electrotonic effect of current flow. This conclusion is strengthened by the observation that the neurones identified as the GI cells by electrophoretic administration of glutamate were also inhibited consistently when the sections were perfused with medium containing 0.1–0.3 mM glutamate.

Fig. 1 Responses of cerebellar interneurons to glutamic acid administered to nearby points. *a*, Recorded from a GI cell. Electrophoretic currents were administered during the periods indicated by the solid lines beneath the records. Intensities of the currents are shown above the records. *b*, Recorded from a GE cell. Solid line: the retaining current of 25 nA was turned off.



The firing of the GI cells was suppressed not only by glutamate but also by GABA. In the experiment illustrated in Fig. 2, the extension of the area in which GABA administration effectively suppressed the firing of a GI cell (GABA-sensitive area) was compared with the extension of the glutamate-sensitive area defined similarly. If GABA inhibits a GI cell directly and glutamate works through mediation of inhibitory interneurons, it would be expected that the extension of the GABA-sensitive area would not coincide with that of the glutamate-sensitive area. In all six GI cells studied, however, both areas almost agreed with each other (Fig. 2). Inhibition by glutamate was not blocked by strychnine (0.5 mg l^{-1}) or picrotoxin (50 mg l^{-1}), the latter being found to block the action of GABA. Although inhibition by GABA was blocked in the medium in which Cl^- was substituted by propionate ions, inhibition by glutamate was not affected in Cl^- -free medium. Thus inhibition by glutamate is not mediated by GABA-releasing neurones. Moreover, inhibition by glutamate was maintained in the medium containing 16 mM Mg^{2+} , in which synaptic transmission in brain slices was reported to be completely blocked⁶. This result excludes the possibility that interneurons intervene in the inhibition by glutamate.

In the cerebellar sections used in these experiments, individual neurones in the granular layer were not distinctly visible under a microscope. Therefore, the GI cells have not

been identified morphologically. At least some of the GI as well as GE cells, however, seem to be large Golgi cells, because action potentials exceeding 5 mV in amplitude were sometimes recorded from the GE and GI cells with electrodes of relatively large tips. When an electric shock was applied to the white matter, the GI cells were inhibited and the GE cells were activated. This suggests that the distinction between the GE and GI cells is not only of pharmacological but also of physiological significance.

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Received June 1; accepted July 6, 1976.

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Growth of transplanted monoaminergic neurones into the adult hippocampus along the perforant path

CENTRAL and peripheral monoaminergic neurones have been found to survive transplantation to certain sites in the adult rat brain¹. The neurones grow extensively in their new location and the newly formed axons extend through the surrounding scar tissue into the host brain. We now report experiments in which transplants of adrenergic, dopaminergic or indolaminergic neurones were transplanted to a cavity in the retrosplenial cortex to give them the opportunity to grow along the lesioned temporoammonic perforant path into the partly denervated hippocampal formation. The results demonstrate a remarkable ability of the regenerating axons to establish reproducible and characteristic terminal patterns in large areas of the hippocampus.

As described previously¹, transplantation involved the placement of pieces of embryonic brainstem—containing major noradrenaline (NA), dopamine (DA), or indolamine (IA) cell groups—or a piece of a mature sympathetic ganglion in a resection cavity in the occipital pole¹, in contact with or in proximity to the dorsal hippocampus (Fig. 1). The operation involved a major portion of the perforant path fibres innervating the dorsal part of the hippocampal complex^{2–5}. Four types of material were transplanted: (1) a half to a third of the superior cervical

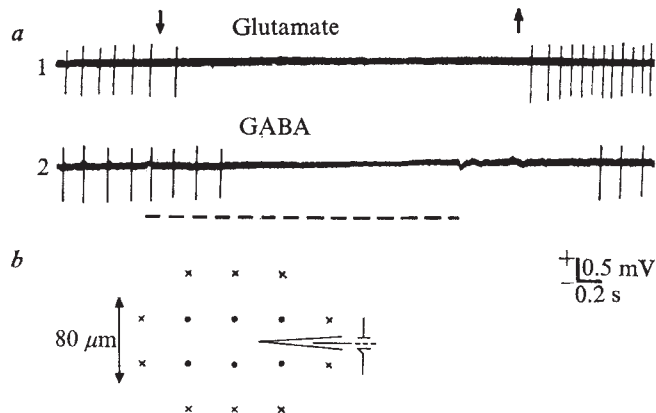


Fig. 2 Suppression of firing of a GI cell by glutamic acid and GABA. *a*: 1, the tip of a pipette filled with 1 M sodium L-glutamate, $\text{pH } 8.2$, touched a point indicated by dots in (*b*) approximately at the time marked by an arrow pointing downwards. The approximate time at which the pipette was removed is marked by an arrow pointing upwards. The retaining current of 25 nA was passed continuously through the pipette. 2, GABA was administered to the same point with an electrophoretic current of 25 nA during the period indicated by a broken line. *b*, Glutamate and GABA inhibited the cell when administered to the points indicated by dots but had no effect when administered to the points indicated by crosses.

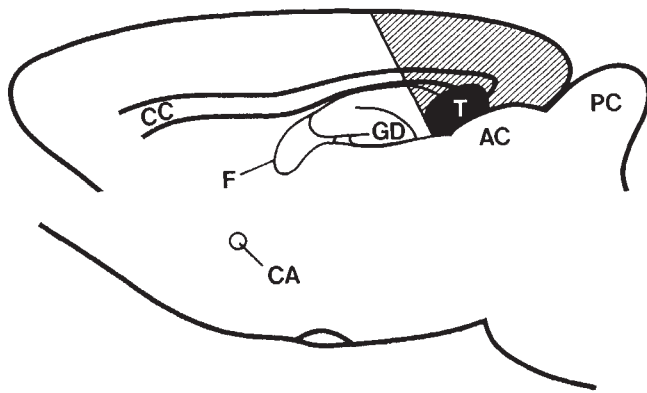


Fig. 1 Position of the neural implants in the hippocampal region, as illustrated in a sagittal plane. Hatched area denotes the extent of the resection cavity. The transplant (T) was placed, under visual control, on to the richly vascularised pia covering the underlying colliculi. The lesion involved part of the entorhinal, retrosplenial and occipital cortex, and the dorsal part of the subicular complex. All animals were subjected to complete intracranial sympathectomy before or at the time of transplantation. AC, Anterior colliculus; CA, anterior commissure; CC, corpus callosum; F, hippocampal fimbria; GD, dentate gyrus; PC, posterior colliculus.

ganglion taken from the adult recipient rat (autologous transplantation); (2) the ventromedial mesencephalon taken from embryos, containing primarily DA neurones; (3) the locus coeruleus region of the pons, taken from embryos, containing primarily NA neurones; (4) the pontine raphe region, taken from embryos, containing primarily IA neurones. These pieces of central nervous system (CNS) were dissected from embryos with a crown-rump length of 15–25 mm, essentially as described by Olson and Seiger⁸,

and they were transplanted homologously into adult rats (Sprague-Dawley; 180–200 g body weight).

The animals were killed 1–6 months later. To remove the normal monoaminergic innervations of the hippocampal formation, about half the recipients were given an intraventricular injection of either 6-hydroxydopamine (6-OHDA; 250 μ g) or 5,7-dihydroxytryptamine (5,7-DHT; 150 μ g) 2–6 d before transplantation. Brains were processed for fluorescence histochemistry according to the Falck-Hillarp formaldehyde method (for technical details, see ref. 7). Animals bearing IA transplants were treated with the monoamine oxidase inhibitor nialamide (300 mg kg⁻¹) 3–5 h before killing, to facilitate visualisation of fibres containing 5-hydroxytryptamine (5-HT).

There were up to about 200 surviving, fluorescent neurones in the peripheral NA transplants, and up to about 500 in the central NA, DA and IA transplants (see ref. 1 for further details of fluorescence microscopical features of the transplants). Of the 18 ganglionic transplants studied, all survived except one, and 26 of the 33 CNS transplants survived. Abundant fibre outgrowth was demonstrable from all four types of transplant. In 19 out of the 26 surviving CNS transplants and in 16 of 17 surviving ganglionic transplants the outgrowing fibres grew into the hippocampal formation, above all the dentate gyrus. The transplantation lesion involved the dorsal presubiculum and subiculum, and a minor adjoining portion of CA 1. In some cases there was also a minor lesion in the dentate gyrus (Fig. 2a and b). Consequently, the outgrowing nerve fibres grew towards a largely intact dentate gyrus and towards the cut surface of CA 1, as shown semischematically in Fig. 2. There was an obvious preferential orientation of the newly formed fibres towards the dentate gyrus; this was most clearly demonstrated when the transplant was placed some distance from the dentate, as in Fig. 2a and c. In such cases the fibres had grown through the surrounding scar tissue for up to

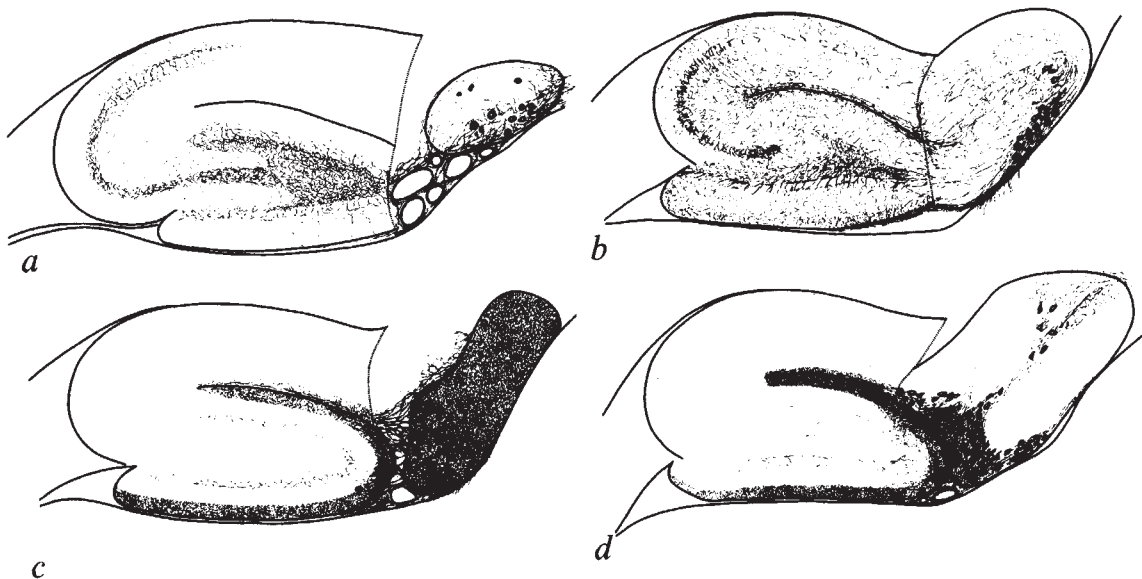


Fig. 2 a–d, Semischematic drawings, in the sagittal plane, of the fibre outgrowth from four types of neural transplants towards the hippocampus (compare Fig. 1). a, Superior cervical ganglion, 6 months survival. The recipient rat was given 250 μ g 6-OHDA, intraventricularly, 2 d before transplantation. The transplant, which underwent considerable atrophy, contained about 90 ganglionic nerve cells with a fairly normal and healthy fluorescence microscopical appearance. This animal had also a minor lesion of the fimbria, which probably caused some increase in the fibre ingrowth. b, NA transplant taken from an embryo with a crown-rump length of 25 mm, 3 months survival. The normal adrenergic and indolaminergic innervations of the hippocampus had been removed with an intraventricular injection of 150 μ g of 5,7-DHT, 6 d before transplantation. The transplant, which retained approximately its original size, contained about 450 surviving catecholamine-containing neurones confined to two major cell clusters. c, IA transplant taken from an embryo with a crown-rump length of 15 mm, 3 months survival. The transplant was filled with a dense pattern of newly formed IA fibres. Because they obscured many of the surviving IA cell bodies it was not possible to assess their number. d, DA transplant from an embryo with a crown-rump length of 20 mm, 6 weeks survival. The normal hippocampal adrenergic and indolaminergic innervations had been removed by a 5,7-DHT injection 3 d before transplantation. The transplant, which probably had increased somewhat in size, contained several hundred surviving fluorescent catecholamine neurones, intermingled with a dense plexus of locally ramifying DA fibres.

1–1.5 mm in rostral, lateral and ventrolateral directions to reach the adjacent dorsal part of the hippocampus and the dentate gyrus. In those specimens where the fibres had failed to reach the hippocampus the transplant had either been displaced more than 1–1.5 mm away from the dentate gyrus, or (in the case of CNS tissue) the surviving monoamine neurones were separated from the dentate by 1 mm or more of transplanted tissue devoid of monoamine neurones.

The fibres grew into the dentate gyrus either along the shortest course straight into the dentate, or along the hippocampal fissure into the dentate gyrus and the hippocampal CA 1 and CA 3 regions. In some cases the fibres also passed for some distance along the pia of the ventral surface of the dentate gyrus before entering the underlying tissue. Although the amount of fibres that grew into the hippocampal formation from the transplant varied from specimen to specimen, their patterning was remarkably reproducible and showed distinct and consistent differences among the several types of grafted neurones.

The peripheral NA neurones had formed a dense varicose terminal pattern in the hilar region as illustrated in Fig. 2a

back to the NA transplant (Fig. 2b). In the dentate gyrus these fibres had—like the normal adrenergic innervation—their densest distribution in the hilar region. From here fibres could be traced through the granular layer into the molecular layer where irregularly running fibres were found throughout its width (Fig. 3c). In some specimens, such as the one in Fig. 2b, the ingrowing fibres tended to aggregate in the outer part of the molecular layer. The fibres reached CA 3 and CA 1 either from the hilus or along the hippocampal fissure. The magnitude of ingrowth into these areas varied considerably. In the richest specimens (Fig. 2b) the new fibre supply covered approximately the dorsal two-thirds of the hippocampus. There was a marked difference between the CA 3, which demonstrated a fairly rich fibre supply in all layers, and the CA 1, where the ingrowing fibres were only sparse and scattered.

The distribution of fibres growing from the central IA transplants (observed in both non-pretreated and in 5,7-DHT-pretreated recipients) was different from that of the central and peripheral NA fibres. The IA transplants formed a layer of densely packed varicose fibres in the outer half of the molecular layer in the dentate gyrus

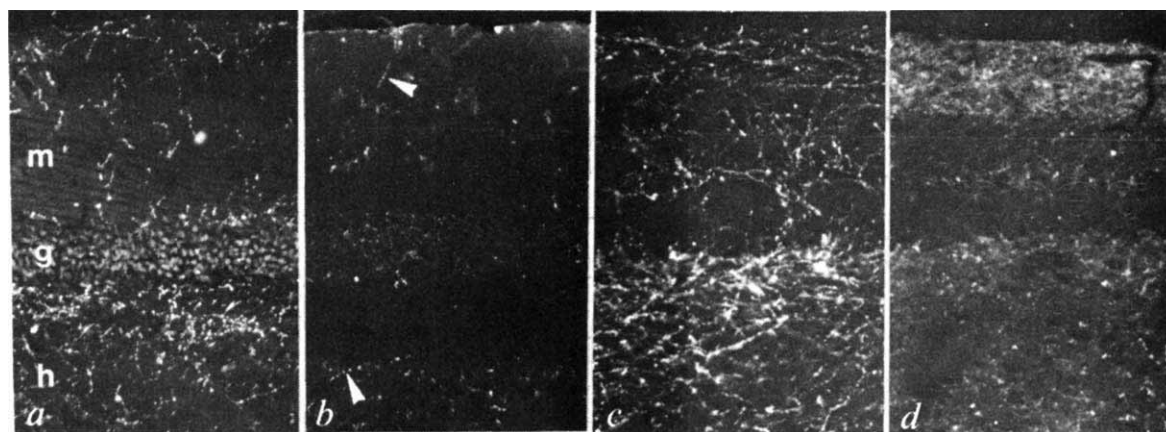


Fig. 3 Detail of the dorsal part of the dentate gyrus showing: a, normal adrenergic innervation in a control rat; b, adrenergically denervated dentate gyrus in a rat treated with 150 μ g of 5,7-DHT (taken from the contralateral, control side of the transplanted rat shown in (c)). Only some single, scattered terminals remain (arrows). c, Patterning of adrenergic fibres originating from a NA transplant, 3 months survival (the dentate was adrenergically denervated before transplantation, as shown in (b)). d, Patterning of indolaminergic fibres originating from an IA transplant, 3 months survival. m, Molecular layer; g, granular layer; h, hilus. Compare Fig. 2. ($\times 90$)

for a specimen taken 6 months after transplantation. Only a few fibres had passed through the granular layer into the basal part of the molecular layer. The outer part of the molecular layer was consistently devoid of fibres. Growth into the hippocampus proper was much more sparse. The fibres appeared to reach the CA 3 region from the dentate gyrus, and in most specimens fibres occurred only in the part of CA 3 close to the dentate. In the 6-month specimen (Fig. 2a) the ganglionic fibres had grown through CA 3 into part of the CA 1 region. The patterning of the ganglionic fibres was remarkably similar to that of the normal adrenergic nerve supply (Fig. 3a, compare refs 8 and 9). These fibres were identified as coming from the transplant by observations with recipients in which the normal adrenergic innervation of the hippocampal formation had been removed by pretreatment with 6-OHDA. The completeness of the denervation was checked in the contralateral hippocampus.

The growth of fibres from the transplanted central NA neurones was studied in rats pretreated with 5,7-DHT. This removed both the normal NA and 5-HT innervations of the hippocampal complex, and the denervation persisted for at least 6 months (Fig. 3b). Within 1–3 months of transplantation the dorsal part of the dentate gyrus and, in some specimens, the hippocampal CA 3 and CA 1 regions received a new fluorescent fibre supply that could be traced

(Figs 2c and 3d). Occasionally, fibres were seen to have grown along the hippocampal fissure to form a similar fibre band in part of the stratum lacunosum-moleculare in CA 1 (Fig. 2c). The position of these fibre bands seems to conform to the normal distribution of the terminals of the perforant path fibres^{4,5,10}. The fibre supply to the lower half of the molecular layer and the granular layer was much more sparse. This was the case also in the hilar region where usually the densest 5-HT innervation is found (compare ref. 11). The IA fibres were not seen to extend into CA 3.

The fibres from the central DA transplants had a characteristic delicate and fine varicose appearance (similar to that of normal central DA terminals) and they were thus well distinguishable from the normal NA innervation also seen in the non-pretreated animals. Like the IA fibres, the outgrowing DA fibres formed a dense, fine varicose fibre band in the outer half of the molecular layer in the dentate gyrus. Figure 2d illustrates a case with an unusually large number of surviving DA neurones, where the ingrowing fibres had covered the entire dorsal dentate. Moreover, a dense band of DA fibres had been formed also in the stratum lacunosum-moleculare of CA 1. The inner part of the dentate molecular layer occasionally had a dense supply in the portion closest to the transplant. Otherwise, only few fibres were observed in the inner part of the molecular

layer and in the hilus, and none was found in the hippocampus proper.

The transplanted central and peripheral neurones were allowed to grow towards a hippocampus that had been partially deafferented through a lesion in the perforant path system and by removal of the monoaminergic brainstem afferents. From the transplant, located in the resection cavity in the retrosplenial cortex, the growth of the new fibres was directed along a course roughly corresponding to that of the lesioned perforant path fibres (compare refs 2–5). Entering the hippocampus through presubiculum and subiculum, the perforant path fibres are known to run through and along the hippocampal fissure to terminate heavily in the outer part of the molecular layer in the dentate gyrus. In interpreting our data, it seems that the outgrowing fibres of all different monoamine neurone types will find their way into the dentate gyrus along the degenerated perforant path, and that two of them, the IA and DA neurones, will distribute their fibres in a manner strongly resembling that of the perforant path fibres. The central and peripheral NA fibres, on the other hand, pattern themselves differently, the most heavy termination being in the hilar region, just as the normal adrenergic innervation. This growth process can be viewed as an attempt at a heterologous reinnervation of the dentate gyrus by regeneration of peripheral adrenergic neurones or by outgrowth of developing central neurones. Contrary to the traditional view that the scar tissue and the composition of the CNS tissue prevent axonal outgrowth in the adult mammalian brain^{12–14}, the axons of the transplanted neurones grew without any apparent obstacle up to at least 1 mm through the developing scar tissue, through the pial covering in the hippocampal fissure, and penetrated deeply into the dentate gyrus and the hippocampus. Here the newly formed fibres extended for distances of up to 3–4 mm from the transplant.

The most intriguing feature of this process is that different types of fibres, although growing along the same route, form distinctly different and highly consistent patterns in the hippocampal formation. This strongly suggests that directional and organising forces—not unlike those demonstrated in lower vertebrates^{15–17}—are operating also in the brains of adult mammals. And in view of the fact that such biochemically closely related neurones as the DA and NA neurone types pattern themselves entirely differently, it seems that these mechanisms have a high degree of precision and selectivity. Nothing is known so far about the ultrastructural mode of termination of the outgrowing fibres. But on the basis of their light microscopic distribution it seems possible that the transplanted IA and DA neurones (and to some minor degree perhaps also the central NA neurones) had grown to fill the terminal space vacated by the lesion of the non-monoaminergic perforant path, and that the adrenergic neurones had regenerated a fairly normal innervation pattern. This is in line with the idea that mechanisms adequate for the re-establishment of severed fibre connections are present in the mammalian brain, but that these mechanisms are not entirely specific for the appropriate neurone type.

This work was supported by grants from the Swedish MRC, Trygg-Hansas fond för personskadeforskning and the Åke Wiberg and Gross foundations.

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Received May 10; accepted July 6, 1976.

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Progesterone may act at hypothalamus and pituitary by way of enhancement of oestrogen retention

PROGESTERONE injection given 24 h or more before ovulation inhibits the ovulatory cycle^{1–5}. There is evidence that progesterone can act directly on the hypothalamus^{6–9} to block ovulation and on the pituitary to reduce pituitary sensitivity to luteinising releasing hormone^{10,11}. Steroid-sensitive target tissues including hypothalamus^{12,13} and pituitary^{14–16} contain

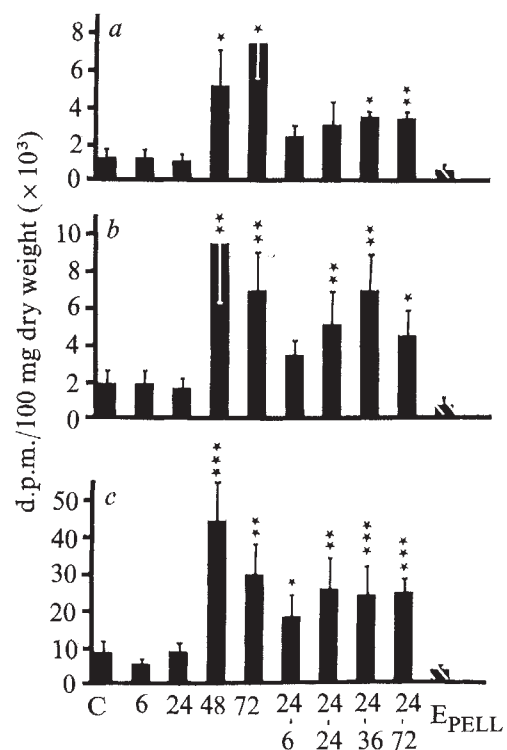


Fig. 1 Adult Sprague-Dawley rats, ovariectomised 14 d previously, received an intraperitoneal injection of 2 μ Ci per 100 g body weight of 2,4,6,7-³H-oestradiol (100 Ci mM⁻¹) in 0.9% NaCl. Previously the animals ($n = 10$) received a 50-mg subcutaneous pellet of progesterone for 6, 24, 48 or 72 h. Other groups had the progesterone pellet removed after 24 h and ³H-oestradiol injected 6, 24, 36 or 72 h later. Saturation of the specific retention system was achieved by subcutaneous implantation of an oestradiol pellet for 72 h (E_{PELL}). Two hours after ³H-oestradiol injections, the animals were killed, perfused with saline and the tissues removed as described previously for hypothalamus¹⁹. Tissues were dried at 60 °C, weighed and then rehydrated in Tris-EDTA. After grinding in all-glass homogenisers extraction was carried out twice with 5 ml ethyl acetate. Combined extracts from a single tissue were dried in scintillation vials. The scintillation cocktail was 0.4% POP and 0.03% POPOP in toluene. Thin lines indicate s.e.m. Student's *t* test was used in the statistical analysis: **P* = 0.05; ***P* = 0.01; ****P* = 0.001. a, Preoptic-anterior hypothalamus; b, arcuate-median eminence; c, pituitary. E_{PELL}, non-saturable oestrogen retention.

a specific oestrogen retention mechanism of limited capacity. Oestrogen-regulated events can be demonstrated only in the presence of a functional retention (receptor) system for oestrogen. In contrast, no specific progesterone retention system has yet been demonstrated for hypothalamus or pituitary. Does progesterone interact with these target tissues in a novel manner? This seems unlikely since other steroids which have been studied—for example, adrenal corticoids¹⁷ and androgens¹⁸—have been found to bind to specific receptors in their target tissues. We therefore tested an alternate possibility that progesterone action on hypothalamus and pituitary might result in modification of the oestrogen receptor system. We tested ³H-oestradiol retention by hypothalamus and pituitary after *in vivo* treatment with progesterone (subcutaneous pellet; release rate = 1.5–3 mg d⁻¹) of various durations from hours to days. *In vitro* ³H-oestradiol-specific retention by oestradiol-receptor complexes transferred to the nuclear fraction of these tissues was also compared for animals receiving previous *in vivo* progesterone treatment compared with ovariectomised animals not receiving progesterone treatment.

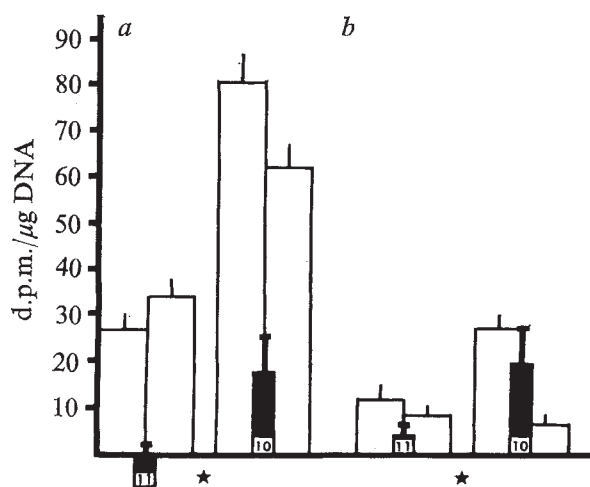


Fig. 2 Adult Sprague-Dawley rats were ovariectomised 14 d before the experiment. Half the animals (right-hand sets of columns) received a 50-mg subcutaneous pellet of progesterone on day 11. The animals were killed by exsanguination. Left-hand sets, controls. After homogenisation of the tissue (0.32 M sucrose, 3 mM MgCl₂, 3 mM K₂MP04, 14 mM 2 mercapto-ethanol, pH = 6.5) one half of each homogenate was incubated at 30 °C for 1 h in 1.0 × 10⁻⁸ M 2,4,6,7-³H-oestradiol. The other half was incubated in the same concentration of ³H-oestradiol plus 1.0 × 10⁻⁸ M unlabelled oestradiol plus 1.0 × 10⁻⁵ M diethylstilboestrol. Nuclear purification followed the methods of Løvtrup-Rein and McEwen²⁰ with slight modification. The nuclear fractions were extracted twice with ethyl acetate and twice with ethanol-diethyl ether (50:50) at 50 °C. Extracts were dried in scintillation vials and counted using 0.4% POP and 0.03% POPOP in toluene. DNA was extracted from the nuclear fraction and measured by the method of Kissane and Robins²¹. a, Hypothalamus; b, pituitary.

Our *in vivo* studies (Fig. 1) show that in the continued presence of progesterone a significant increase in ³H-oestradiol retention has occurred by 48 h and is still expressed at 72 h. The magnitude of this increase was pituitary > arcuate-median eminence > preoptic-anterior hypothalamus. When the animals are exposed to progesterone for 24 h, no change in ³H-oestradiol was observed if the progesterone source was removed at that time; a significant increase in ³H-oestradiol retention was found for pituitary at 30 h, at 48 h in the arcuate-median eminence region, and at 60 h in the preoptic-anterior hypothalamic region. Thus, in the continued presence of the progesterone source, all tissues showed a response at 48 h. When the response was measured after removal of the progesterone source, however, the pituitary showed the shortest response interval

(30 h). These data on *in vivo* total tissue retention are supported by the demonstration that the specific *in vitro* oestrogen retention by the nuclear fraction was significantly enhanced when ovariectomised animals were pretreated with progesterone for 72 h (Fig. 2).

Our study shows that progesterone treatment *in vivo* significantly modified oestradiol retention, studied *in vivo* for whole tissue and *in vitro* for the nuclear fraction from hypothalamus and pituitary of female rats. This supports the concept of progesterone acting both at the neural level⁶⁻⁹ and pituitary^{10,11} level in regulation of the ovulatory cycle. Exposure of the pituitary and the neural tissues to progesterone for 48 h resulted in an increase in specific oestradiol retention. This increase could be observed for at least 72 h after the end of 24 h progesterone treatment. The change in oestradiol retention observed after 24 h of progesterone treatment correlates with the time required for progesterone to inhibit events of the ovulatory cycle.

At present, no nuclear progesterone receptor has been demonstrated for the hypothalamus or the pituitary. Through some mechanism requiring progesterone and at least 24 h, however, the amount of oestradiol receptor found in the nuclear fraction is increased. The possibilities of whether the biological effect of progesterone on the ovulatory cycle is mediated by an increase in oestradiol receptors in these tissues, or whether the increase in oestradiol receptors is caused by a progesterone-mediated block of a later step in the oestradiol-receptor-genome interaction is under investigation.

This work was supported by the USNSF and was carried out with the facilities of the Whitehall Foundation. We thank Mrs Ginger Wang and Mrs Kay Chien for assistance.

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Received February 3; accepted June 8, 1976.

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Combination of insulin chains on an anti-insulin antibody-Sepharose column

WHEN the mixture of products obtained by combining insulin A and B chains in solution (for a review, see ref. 1) was chromatographed on an anti-insulin antibody-Sepharose column² no clear separation was achieved³. From the low biological activity (24.1%) of the material with the same antibody affinity as natural insulin (10.9% of the total bound products) it was concluded³ that resynthesised insulin with authentic structure constituted only a small portion of this fraction. Combination of insulin chains in solution has always involved a number of steps^{4,5} and has produced a mixture of products from which pure active insulin could be isolated by gel filtration and ion-exchange chromatography⁶. Linking the chains using the procedure described in this report, however, has given only one

combination product that, according to various criteria, was identical with insulin and could be separated from unreacted A and B chains in one step.

That the B chain of insulin cross reacts with anti-insulin antibodies, whereas the A chain does not⁷, formed the basis for our new method of resynthesising insulin from the two chains. This method also involved the participation of antibodies (Fig. 1). During resynthesis of insulin from the isolated chains, a major product formed is a polymer consisting mainly of disulphide-linked B chain molecules⁸. Thus, active insulin can be obtained in good yield only if the polymerisation of the B chain is prevented. We assumed that S-sulphonate B chain, when bound to Sepharose-coupled anti-insulin antibodies, could no longer polymerise on reaction with reduced A chain if the distance between the B-chain molecules on the solid matrix was too large for inter-B chain disulphide bridges to form. Furthermore, antibody-bound S-sulphonate B chain was presumably fixed in a conformation identical or nearly identical to that of the B chain in the insulin molecule. This would to a great extent assist the 'parallel alignment' of reduced A chain and the closing of the right disulphide bonds to give active insulin.

Anti-insulin antibodies were prepared by injecting each of six guinea pigs with a suspension of 1.2 mg pig insulin in a mixture of 0.1 M Na_2HPO_4 (0.7 ml, pH 7.21) and Freund's complete adjuvant (0.3 ml). Sera were obtained by heart puncture 30 d after immunisation, and assayed using the immunodiffusion test⁹ against pig insulin. The IgG fraction of strong sera was isolated using the procedure of Levy and Sober¹⁰. The concentration of purified antibodies was 6.4 mg ml^{-1} . To 22 ml of this solution 5 g freshly BrCN-activated Sepharose 4B was

Fig. 1 Combination of insulin chains on Sepharose-coupled anti-insulin antibodies. AIA, anti-insulin antibody; $\text{A}(\text{SH})_4$, tetrathiol A chain; $(\text{S}-\text{S})\text{A}(\text{S}-\text{S})_2\text{B}$, combination product with one intrachain and two interchain disulphide bonds.

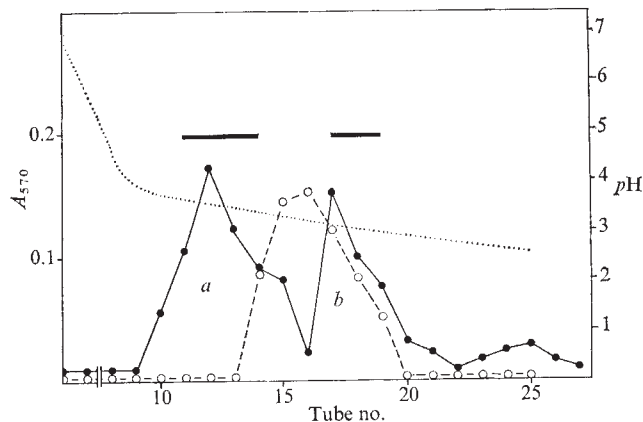
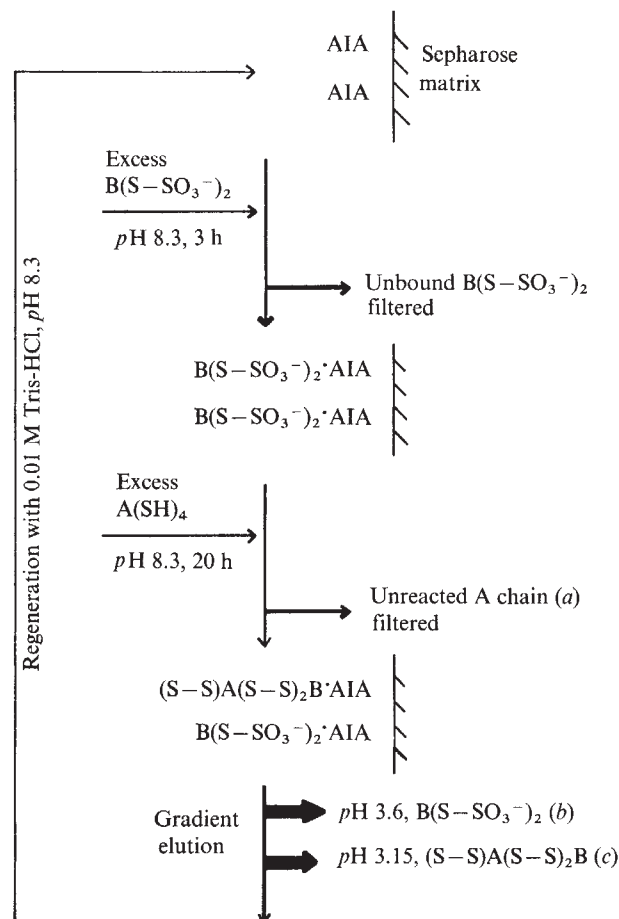


Fig. 2 Gradient elution of natural insulin (○) and recombination products (●) from anti-insulin antibody-Sepharose column ($3.2 \times 1.3 \text{ cm}$). Solvents used were 0.01 M Tris-HCl (pH 8.3, 40 ml) and 1 M acetic acid (pH 2.6, 40 ml)² (volume per tube approximately 1.5 ml). From each tube a 0.5-ml aliquot was removed for alkaline hydrolysis¹³ followed by the ninhydrin reaction. Extinctions were read at 570 nm. By comparison with standard curves the protein content of tubes 11–14 (a, $\text{B}(\text{S}-\text{SO}_3^-)_2$) was found to be 0.159 mg or 44.2 nmol, that of tubes 17–19 (b, insulin) was 0.102 mg or 17.8 nmol. The total amount of B chain bound by the antibody-Sepharose column was 0.076 μmol compared with the maximum capacity of 0.540 μmol . The antibody-Sepharose column was used repeatedly. All elution patterns were identical to that shown above. In later runs $\text{B}(\text{S}-\text{SO}_3^-)_2$ and insulin could therefore be localised by measuring the pH value of each fraction of the eluate.

added, and the antibodies were coupled to the solid support by the method of Axen *et al.*¹¹. After careful washing, an aliquot of the antibody-Sepharose gel was subjected to acid hydrolysis (6 N HCl, 105–110 °C, 24 h) followed by amino acid analysis. This showed that 0.054 μmol IgG was bound per g of Sepharose. Since there are two antigen-binding sites per antibody molecule the maximum capacity of the antibody-Sepharose for insulin or insulin B chain was 0.108 $\mu\text{mol g}^{-1}$.

S-sulphonate chains were prepared from natural insulin and separated on Dowex 50 as described previously⁸. B chain-bi-sulphonate ($\text{B}(\text{S}-\text{SO}_3^-)_2$; 7.65 mg; 2.125 μmol) was added to 5 g antibody-Sepharose in 0.01 M Tris-HCl, pH 8.3 (total volume 10 ml). The mixture was stirred for 3–4 h and then filtered. The gel containing 0.076 μmol antibody-bound B chain was washed thoroughly with the same buffer. From the combined filtrates, unbound $\text{B}(\text{S}-\text{SO}_3^-)_2$ was recovered. Tetrathiol A chain was prepared from $\text{A}(\text{S}-\text{SO}_3^-)_4$ by reduction with mercaptoethanol (100-fold molar excess per $\text{S}-\text{SO}_3^-$ group) in 5 M urea, pH 5. After 16 h a 15-fold amount of acetone containing 1.4% (v/v) 1 N HCl was added to precipitate the reduced A chain. The precipitate was centrifuged

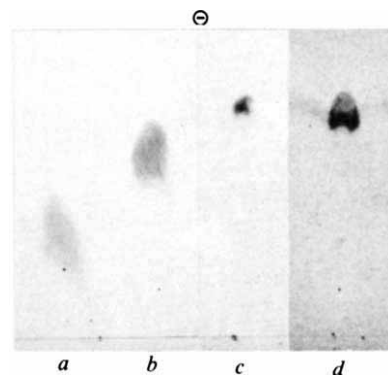


Fig. 3 Electrophoresis of recombination products and natural insulin on cellulose-coated thin-layer plates ($20 \times 20 \text{ cm}$) in 1.2 M formic acid and 2 M urea, pH 2.2, at 600 V for 50 min. The plates were sprayed with Pauly reagent. a, A chain-bi-disulphide; b, unreacted $\text{B}(\text{S}-\text{SO}_3^-)_2$; c, resynthesised insulin; d, natural insulin.

off and dried *in vacuo*. Its sulphhydryl content, determined using the Ellman reagent¹², was found to vary between 3.6 and 3.8 mol per mol reduced A chain. Reduced A chain (4.06 mg, 1.735 μ mol) was dissolved in 0.01 M Tris-HCl, pH 8.3, the solution added to the B(S-SO₃⁻)₂-antibody-Sepharose complex (volume of recombination mixture 12–13 ml) and stirred gently. After approximately 20 h no more sulphhydryl groups were detected. The gel was filtered and washed carefully. Electrophoresis (Fig. 3a) showed that the only product present in the filtrate was A chain which had not reacted with the antibody-bound B chain but had been oxidised to give the bidisulphide derivative.

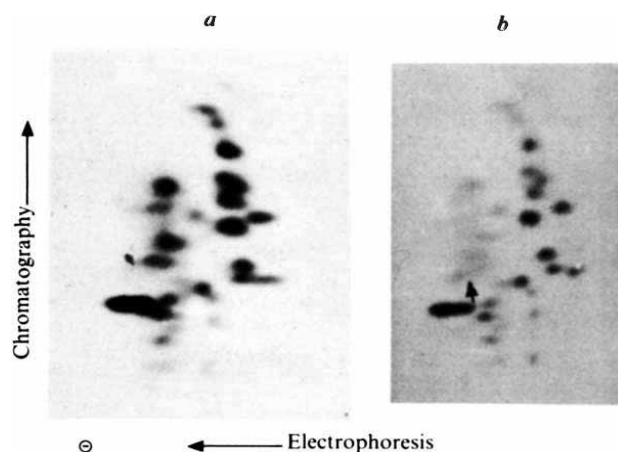


Fig. 4 Peptide maps of peptic digests of natural insulin (a, 0.12 mg) and recombined insulin (b, 0.03 mg)¹⁴. The digestion mixtures were applied on cellulose-coated thin-layer plates (20 × 20 cm) and were resolved by electrophoresis at 600 V for 75 min. in 12% acetic acid (v/v), adjusted to pH 1.9 with formic acid; and by thin-layer chromatography in 1-butanol-acetic acid-pyridine-water (30:6:20:24). The plates were sprayed with ninhydrin solution. The arrow in b marks the two weak spots that were not observed on the peptide map of pepsin-treated natural insulin.

The gel was then placed in a column (3.2 × 1.3 cm) and the products which were linked non-covalently to the antibody-Sepharose complex were eluted by a pH gradient (8.3–2.6), (Fig. 2). The first peak (absorption maximum at pH 3.6) contained unreacted B(S-SO₃⁻)₂ as shown by amino acid analysis and electrophoresis (Fig. 3b). The absorption maximum of the second peak was at pH 3.15 and was close to that of amorphous natural insulin (Fig. 2). The material of peak b was desalted on Biogel P-2 in 0.05 M NH₄HCO₃ (yield, 0.018 μ mol or 23.7% based on the amount of bound B chain) and then submitted to electrophoresis (Fig. 3c), peptic digestion followed by peptide mapping (Fig. 4), and amino acid analysis of an acid hydrolysate. Electrophoretic mobility (Fig. 3), peptide map (Fig. 4) and amino acid composition of product c agreed well with those of natural insulin.

Amino acid ratios were as follows (values reported for pig insulin¹⁵ are in parentheses): Asp 2.7 (3); Thr 1.9 (2); Ser 3.2 (3); Glu 7.0 (7); Pro 1.0 (1); Gly 3.8 (4); Ala 2.8 (2); Cys 5.7 (6); Val 3.6 (4); Ile 1.6 (2); Leu 6.0 (6); Tyr 3.7 (4); Phe 3.0 (3); His 2.1 (2); Lys 1.0 (1); Arg 0.9 (1). In the fat cell assay^{16,17} the product of peak a was inactive whereas the product of peak b (Fig. 2) had an insulin activity of 67%. The effective yield of authentic recombined material was thus 15.9% based on the amount of bound B chain.

Antigenic properties, electrophoretic mobility, and amino acid composition of recombination product and natural insulin were in good agreement. The peptide map of the recombined material showed two additional spots (Fig. 4b, arrow) but was otherwise indistinguishable from that of natural insulin, indicating that very few, if any, wrong disulphide

bridges had formed during the coupling of A and B chains on the antibody-Sepharose matrix. The additional spots on the peptide map and the fact that the recombination product was not fully active may have been caused by the presence of a small amount of antibody eluted from the affinity column as insulin-antibody complex.

The chain combination method described here offers several advantages. Reconstituted insulin which was almost identical to natural insulin was obtained in one step without further purification. Unreacted A and B chains were isolated separately and could be easily reintroduced into the combination reaction. Finally, the antibody-Sepharose column could be used repeatedly. The possibility of using an anti-B chain-antibody-Sepharose matrix has not yet been studied.

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Farbwerke Hoechst AG, Frankfurt (Main), for insulin; Drs K. Rajewsky and K. Eichmann for discussions; and Drs B. Hansen and J. H. Nielsen (Steno Memorial Hospital, Gentofte, Denmark) for carrying out the biological assays.

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Received December 22, 1975; accepted June 24, 1976.

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Increase of glucagon receptors in hyperthyroidism

THYROID hormones influence the lipolytic response of rat adipocytes to adrenaline, glucagon, ACTH and TSH^{1,2}. Krishna, Hynie and Brodie³ provided evidence that hyperthyroidism is followed by an increase in the nor-adrenaline-sensitive adenylate cyclase without any change in phosphodiesterase activity. Subsequent investigations have shown that adenylate cyclase activity measured as fluoride stimulated activity is unchanged in hyperthyroidism⁴ and confirmed that phosphodiesterase activity is normal^{4,5}. Since the amount of hormone-sensitive lipase does not appear to be increased in hyperthyroidism³ these studies indicate an action of thyroid hormones either on the hormone receptors for lipolytic hormones or on the transmission of the hormonal message from the receptors to adenylate cyclase. This prompted the present study in which we found that hyperthyroidism was associated with an increased binding of glucagon to fat cells as well as increased glucagon stimulated production of cyclic AMP and glycerol.

Male Wistar rats (120–175 g) were rendered hyperthyroid by daily subcutaneous injections of 20 μ g triiodothyronine (T3) (Glaxo) for 8 d. Paired litter mates received in the same period injections of the vehicle for T3. Epididymal

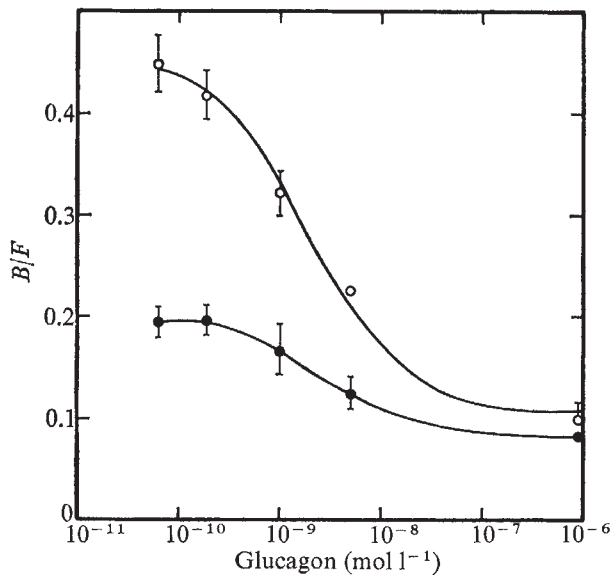


Fig. 1 Binding of glucagon to fat cells from a T3-treated (○) and a control (●) rat. Fat cells ($10 \mu\text{l ml}^{-1}$) were incubated at 37°C in a Krebs-Ringer buffer with 25 mM HEPES (pH 7.4) and 1% of human serum albumin (Behringwerke) with ^{125}I -labelled glucagon or 0.18 nM ^{125}I -labelled glucagon plus unlabelled glucagon to give the indicated concentrations. After 30 min of incubation 6 aliquots of the cell suspension were transferred to microtubes containing approximately 100 μl of silicone oil and the cells were separated from the medium by centrifugation through the oil in a Beckman Model 152 Microfuge at 2,500g for 1 min. After cutting through the oil layer the cells and aliquots of the medium were counted for ^{125}I -activity. The ordinate is expressed as ^{125}I -activity per cells divided by ^{125}I -activity per 1 medium. Bars represent s.d., $n = 6$.

and perirenal fat pads were then removed and suspensions of isolated fat cells prepared⁶.

Binding of glucagon to the isolated fat cells was measured at equilibrium by the displacement of ^{125}I -labelled glucagon (NOVO, Copenhagen, Denmark) by unlabelled glucagon. Cells were separated from medium by centrifugation through a silicone oil and counted for radioactivity in a gamma counter with a background of 3 c.p.m. and an efficiency of 35% (ref. 7). The number of glucagon receptors were calculated from a computerised fit of the experimental data⁷, since the binding of glucagon to the receptor can be described as a reversible binding between the glucagon molecule and a single class of receptors⁸.

Figure 1 shows binding curves for glucagon to fat cells from a hyperthyroid and a control rat. The binding of glucagon was measured in seven consecutive, paired experiments. The mean number of glucagon receptors on fat cells from control animals were 260 ± 161 (s.d.) pmol per 1 cells, $1.8 \times 10^4 \pm 1.5 \times 10^4$ per cell and 1.6 ± 1.1 per μm^2 cell surface; the corresponding values for fat cells from T3 treated rats were 658 ± 331 pmol/1 cells ($P < 0.01$ by a paired two-tailed Student's t test), $2.8 \times 10^4 \pm 1.6 \times 10^4$ per cell ($P < 0.05$) and 3.5 ± 1.8 per μm^2 cell surface ($P < 0.01$). Calculated as percentage of control values, the hyperthyroid fat cells possessed $227 \pm 101\%$ (s.d.) glucagon receptors per 1 cells ($P < 0.01$), $233 \pm 116\%$ per cell ($P < 0.05$) and $255 \pm 100\%$ per μm^2 cell surface ($P < 0.01$). Variations in cell size could not account for the differences between the two groups since mean cell diameter was virtually the same in some experiments. The dissociation constant (K_d) for the binding of glucagon was nearly identical for the two groups investigated (mean value 2.0 nM for controls and 2.2 nM for T3 treated). The degradation of glucagon measured as the ^{125}I -activity in a trichloroacetic acid supernatant was not changed by treatment with T3.

The glucagon stimulated production of cyclic AMP and

glycerol was measured in order to study if the increased binding was reflected in the physiological response to the hormone. Figure 2 shows the correlation between the amount of glucagon receptor per 1 cells and the amount of cyclic AMP measured in the presence of 10^{-6} M glucagon. There is a highly significant correlation between these two parameters ($r = 0.877$, $P < 0.001$). Figure 3 shows the correlation between the amount of glucagon receptors and the production of glycerol in the presence of 10^{-6} M glucagon ($r = 0.838$, $P < 0.001$).

These results suggest that the increased glucagon stimulated production of cyclic AMP and glycerol in hyperthyroid fat cells is a consequence of an increased binding of hormone. The increased biological response to other lipolytic hormones might be explained by a similar increase in the number of receptors. To investigate whether T3 could also exert an effect on the binding of non-lipolytic hormones, we studied the specific binding of insulin, measured at a concentration of 0.7 nM insulin, in the same experiments where binding of glucagon was determined. The amount of specifically bound insulin was correlated to the amount of glucagon receptors ($r = 0.816$, $P < 0.001$). This increase in binding of insulin concomitant with the increase in glucagon receptors occurred without changes in the unspecifically bound insulin, and without changes in the K_d for insulin in three experiments where this was determined.

As intravenous injection of glucagon is followed by a supranormal rise in plasma cyclic AMP in patients with hyperthyroidism^{9,10} and adrenaline causes supranormal urinary excretion of cyclic AMP in these patients¹¹, it seems reasonable to suppose that the observed effect is not confined to the glucagon and insulin receptors of fat cells from experimentally hyperthyroid rats.

Rosenqvist¹² demonstrated that the catecholamine receptors of fat cells from hypothyroid patients changes from

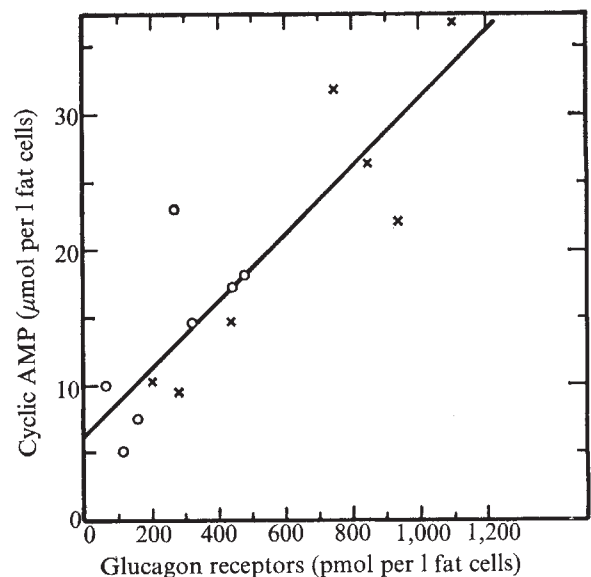


Fig. 2 Relationship between the calculated number of glucagon receptors and the glucagon stimulated cyclic AMP production. Fat cells from T3-treated (x) or control (○) rats were incubated at 37°C in a Krebs-Ringer buffer with 25 mM of HEPES (pH 7.4), 1% human serum albumin, and 2.5 mM of theophylline in the presence of 10^{-6} M glucagon. After 330 s the incubation was stopped by addition of an equal volume of boiling EDTA (20 mM) and the mixture was boiled for another period of 330 s. Cyclic AMP was determined by a competitive protein binding assay¹³. The number of glucagon receptors was calculated from binding curves as shown in Fig. 1. The correlation coefficient between the two parameters is 0.877, $P < 0.001$.

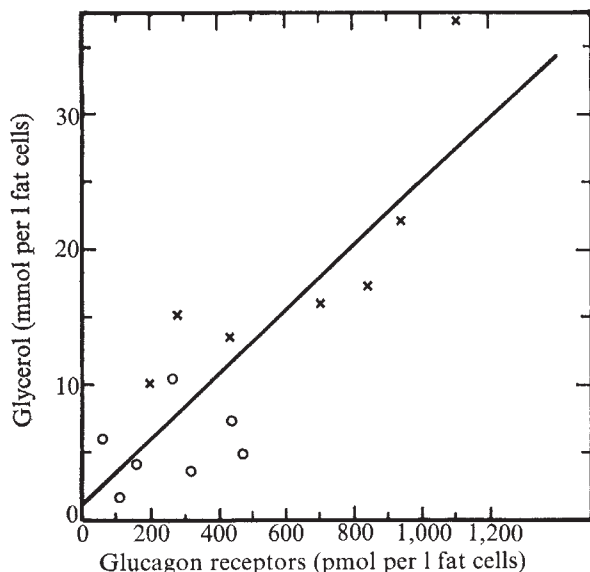


Fig. 3 Relationship between the calculated number of glucagon receptors and the glucagon stimulated production of glycerol. Fat cells from T3-treated (×) or control (○) rats were incubated for 60 min. at 37 °C in a Krebs-Ringer buffer containing 25 mM HEPES (pH 7.4), 2% of human serum albumin and 10^{-6} M glucagon. The incubation was stopped by addition of double volumes of 87 mM ZnSO_4 and 83 mM Ba(OH)_2 . Glycerol was determined by a double enzymatic fluorometric assay¹⁶. The number of glucagon receptors was calculated from binding curves as shown in Fig. 1. The correlation coefficient between the two parameters is 0.838, $P < 0.001$.

predominantly β adrenergic to α adrenergic characteristics, and similar findings have been reported in the hearts of hypothyroid rats¹³. These results and the present study suggest that thyroid hormones are important regulators of the properties of hormone receptors located to the plasma membrane. Other examples of hormonal regulation of hormone receptors has been discussed by Tata¹⁴.

This investigation was supported by a grant from the NOVO foundation.

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Simultaneous detection of two cell populations by two-colour fluorescence and application to the recognition of B-cell determinants

CELL separation techniques, the possibility of recognising different markers by immunofluorescence and functional tests, have subdivided cells once thought to be a homogeneous group into different subclasses. This development has been most prominent for the so-called mononuclear cells. A difficulty inherent in the classification of such subclasses (of, for example, mononuclear cells) is that it is often almost impossible to determine precisely what percentage of the cells belongs to a certain subclass, and whether the sum of the subclasses is equal to, lower than or even higher than 100% of the total of the cells analysed. A typical recent example was the identification of the “null” or K cell^{1,2}. Such analysis is greatly facilitated if we analyse a cell suspension stained with two or more different fluorescent dyes visible at the same time. This is feasible.

With the use of a monochromatic 50-W light source (HBO 50 W/a.c.) an LP455 and SP490 excitation filter combination, an FT510 dichroic mirror and LP520 barrier filter, the green fluorescence of isothiocyanate and the red fluorescence of rhodamine or ethidium bromide become visible simultaneously.

We have exploited these techniques to develop a rapid, simple and sensitive method of recognising polymorphism of B-cell determinants. Interest in these B-cell determinants (which are called Ia determinants in the mouse) is growing rapidly. They may be directly involved in T-B cell interaction in the immune response^{3,4}, code for disease predisposition⁵ and the MLC-stimulating determinants⁶ and determine allograft survival⁷. Most information on the genetics and possible biological importance of these B-cell determinants has been collected in the mouse⁸, but information in man⁹ and the rhesus monkey⁹ is rapidly growing. Although B-cell determinants were identified virtually at the same time in the mouse and in man^{10–13}, the study of these antigens in man was hindered by the lack of sera and, especially, appropriate techniques to detect them. For routine work in man only peripheral blood lymphocytes can be used and here the proportion of B cells can be as low as 5–10%. Although the original enriching process by T-cell rosetting with SRBC and Ficoll-Isopaque gradient centrifugation¹⁴ has been improved by the addition of papain or dextran (ref. 15 and W. Bodmer, personal communication), it remains (especially if the starting material contains a low percentage of B cells) a cumbersome, time-consuming and not fully predictable procedure. For this reason, a modifica-

Table 1 A comparison of the long B-cell complement-dependent cytotoxicity test¹⁴ and the two-colour B-cell test

	Long B-cell cytotoxicity			Total
	Positive	Negative		
Two-colour B cell	Positive	58	10	68
	Negative	9	103	112
		67	113	180
				χ^2 (Yates) = 104.8
				$P = < 0.000001$

These data illustrate the excellent agreement between the two techniques and provide also information on the reproducibility of B-cell typing. The discrepancies were mainly caused by 2 of the 15 sera tested and were partly due to anti non-B cell cytotoxicity which cannot be recognised in the long B-cell complement-dependent cytotoxicity test.

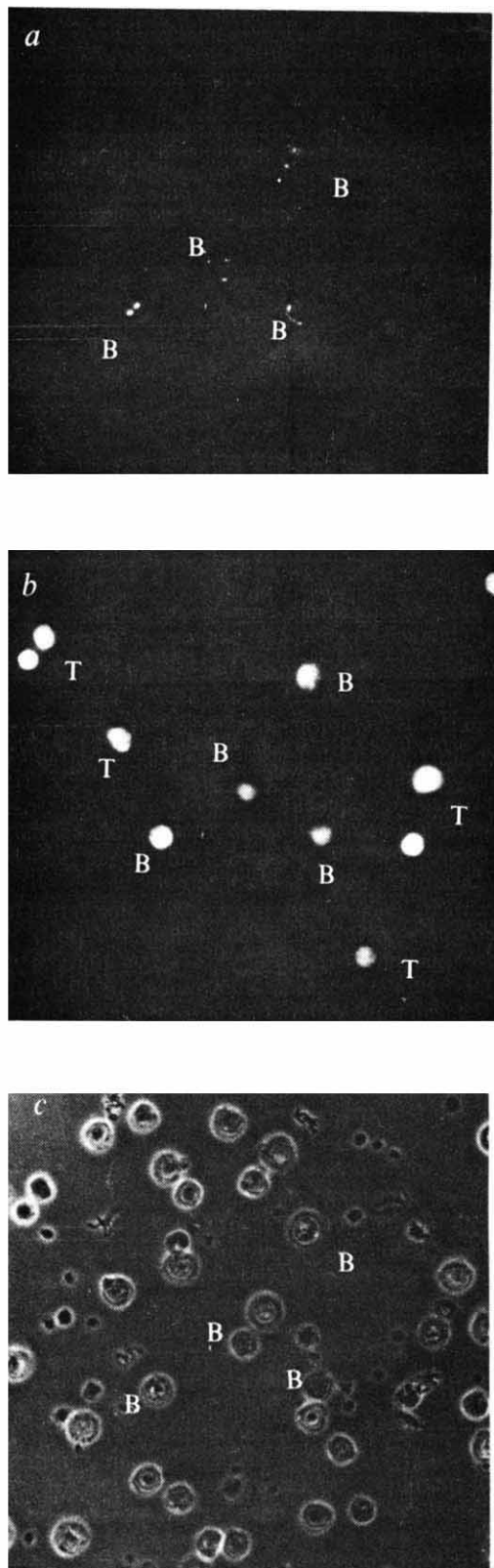


Fig. 1 Picture been taken of the green fluorescence of the immunoglobulins separately (a), from the red fluorescence of the nucleus (b). Note that also some non-B cells (presumably T cells) have been killed. The green and red fluorescence are depicted separately so that they can be seen in black and white only. When the test is read they are visible at the same time. c, The cells visible in phase contrast; many more cells were present than are visible in fluorescence.

tion lacking many of the drawbacks of the previously published method using two-colour fluorescence is described here.

In short, mononuclear cells were isolated from peripheral blood, the B cells were stained by fluorescein-conjugated anti-immunoglobulin and these so identified B cells (together with the unlabelled T and K cells) were incubated with anti-B-cell antibody and complement. Killed cells were stained by the addition of ethidium bromide. The exact procedure was as follows: 4 ml of heparinised blood was diluted twice in phosphate-buffered physiological saline (PBS) and 4 ml of this suspension was carefully layered on Böyum type Ficoll-Isopaque gradient¹⁶. The tubes were spun for 15 min at 1,000g and the mononuclear cells were collected from the interphase, washed 3 times in phosphate-buffered saline containing 1% bovin serum albumin (PBS-BSA 1%). Cells (4×10^6 – 6×10^6) suspended in 0.5 ml PBS+BSA 1% were incubated with 2 drops (0.05 ml) of goat anti-human immunoglobulin diluted 1:3 (Behringwerke AG, Marburg/Lahn, Germany) for 5 min at 37 °C. After three washings with PBS-BSA 1% the cells were adjusted to $8,000 \text{ mm}^3$. A sample (0.5 μl) of platelet-absorbed anti-B-cell serum was incubated in a Möller-Coates A/S tray (Moss, Norway) under paraffin oil with 0.5 μl of mononuclear cell suspension (4,000 cells) for 60 min at 20 °C. After that 2.5 μl fresh rabbit serum was added to provide an excess of complement factors and incubated for 2 h at room temperature. Finally, 0.5 μl of a 1:70 dilution of a stock solution ($100 \mu\text{g ml}^{-1}$) of ethidium bromide (Sigma) dissolved in EDTA 5% in saline was added. After putting a coverglass on the tray the reading was done on an inverted Leitz or Zeiss microscope with an epi-illuminator. Figure 1a shows a picture of the green fluorescence of the immunoglobulins, Fig. 1b the red fluorescence of the killed cells and Fig. 1c the corresponding phase-contrast picture. Note that also some non-B cells (presumably T cells) were killed.

The two-colour B-cell cytotoxicity technique was compared with the results obtained with the long B-cell cytotoxicity technique previously described. Excellent agreement was found between the two techniques (Table 1).

The red fluorescence of ethidium bromide can be strongly enhanced by switching the filterset in the two-wavelength vertical illuminator to green excitation (Table 2), which can be done with the new Leitz and Zeiss type of two-wavelength illuminators after Ploem quite easily¹⁷. It is thus possible to use about 4–8 times lower concentration of ethidium bromide than described above for blue excitation only. By using these lower ethidium bromide concentrations and the two-wavelength excitation method with subsequent excitation with blue followed by green light, it is also possible to make black and white micrographs for documentation. With green excitation of the same cell the red fluorescence of the (dead) nucleus is recorded. This two-wavelength procedure is also promising for future automation of this method since it provides sequential images which can be counted more easily.

As Fig. 1 shows, an advantage of the two-colour method is that it not only indicates whether a part of the mononuclear cells from peripheral blood is killed, but also whether there are B cells or also non-B cells killed. As a matter of fact, in our first trials with the new technique, two sera were identified which were thought to contain only anti-B-cell antibodies but could be shown to contain what looked like antibodies recognising a T-cell polymorphism as well. The method seems to be reliable and reproducible. Using the two-colour fluorescence method it was thus possible to facilitate greatly the recognition of the polymorphism of B-cell determinants. The advantage of this new method compared with the long B-cell complement-dependent lymphocyte toxicity technique is that it takes about 6 h (inclusive of reading) against 12 h for the

Table 2 Filter combinations for fluorescence microscopy of mononuclear cells stained with FITC conjugate and ethidium bromide

Combination	Excitation wavelength (nm)	Excitation filters	Barrier filter	Colour and strength of fluorescence	
1	Blue	LP ^x 455-SP ⁺ 490	LP520	FITC green: Ethidium bromide (higher concentration) red:	+++
2	Blue	LP455-SP490	LP520-SP560	FITC green: Ethidium bromide (lower concentration) greenish:	+++ ±
3	Green	LP515-SP560	LP590	FITC: Ethidium bromide (lower concentration) red:	± ++++

LP^x: long-wave pass filter; SP⁺: short-wave pass interference filter (SP = German KP).

old method; and that one can be certain that if a fraction of the cells are killed, these cells are indeed B cells.

The point of more general importance is, however, that this approach makes possible the direct identification of two (or three) cell subclasses in one preparation. It is expected that this will improve the accuracy of the determination of the fraction of the different subclasses in cells only recently thought to be homogeneous.

This work was in part supported by the NIH, the Dutch Organization for Health Research (TNO), the Dutch Foundation for Medical Research (FUNGO) which is subsidised by the Dutch Organization for the Advancement of Pure Research (ZWO), and the J. A. Cohen Institute for Radiopathology and Radiation Protection (IRS).

Note added in proof: After completion of this letter it was pointed out to us that two-colour fluorescence has been described previously^{18,19}. We compared our approach with that previously described and found that only our filter combination and protocol makes it possible to detect the B-cell antigens satisfactorily, because resolution was significantly better.

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Received June 7; accepted July 2, 1976.

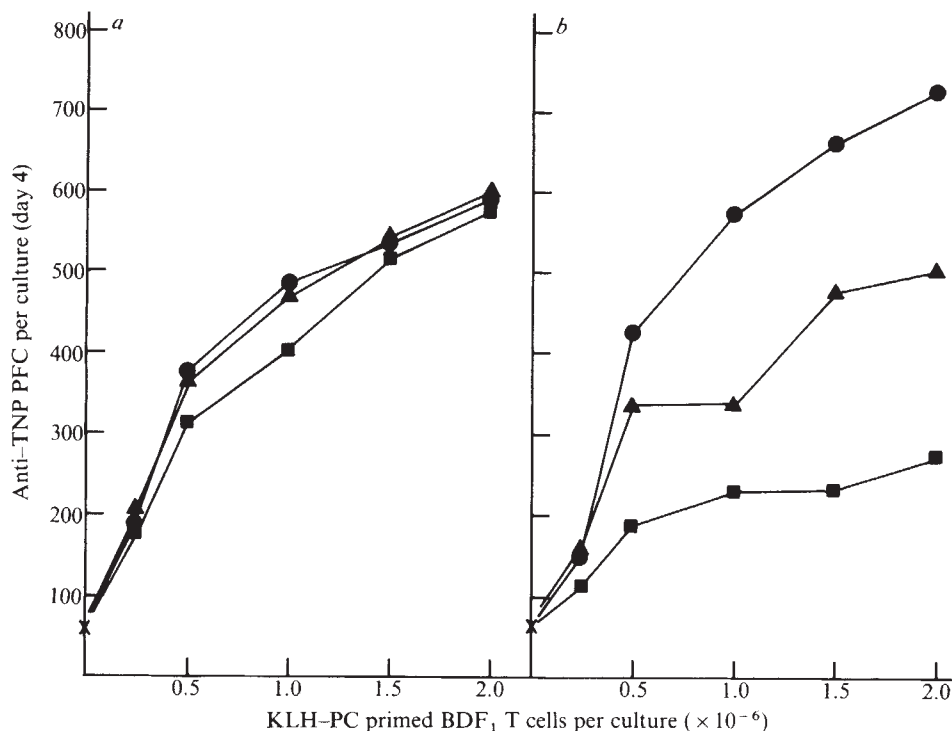
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Helper T cells recognise antigen and macrophage surface components simultaneously

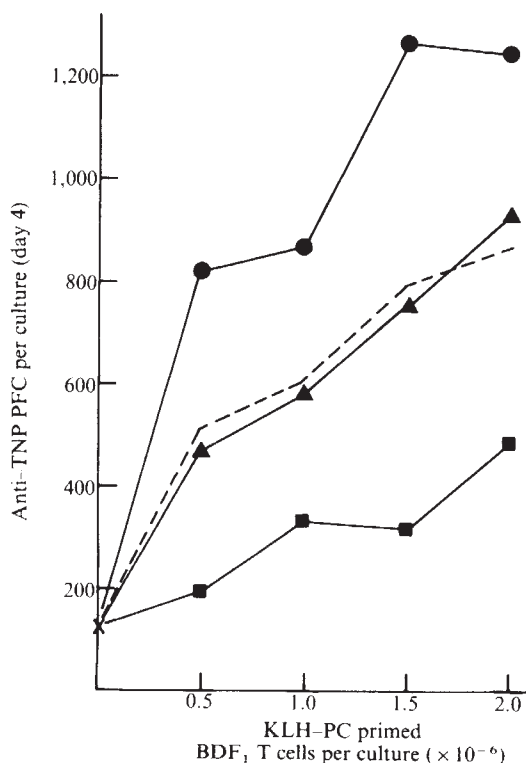
ALTHOUGH the T-cell antigen receptor has been shown to be as highly specific for antigen as the immunoglobulin receptor of B cells, considerable evidence suggests that genes other than those which code for immunoglobulins are involved in the recognition of antigen by T cells. Research in this area has centred on the role of gene products of the major histocompatibility complex (MHC), in particular of the I region of the H-2 complex of mice, in the antigen-specific receptor of T cells¹⁻³. Other evidence has suggested that the specificity of T cells is determined by the simultaneous recognition of antigen and MHC cell-surface components. The best evidence for this comes from experiments with mice in which T-cell cytotoxic effectors specifically raised to the H-Y (ref. 4) or other minor histocompatibility antigens⁵, viral antigens⁶ or the hapten trinitrophenol (TNP)⁷ were shown to recognise simultaneously both the antigen and the products of the *D* or *K* locus of H-2 expressed on the target cells. Investigations of the role of MHC gene products in the antigen-mediated interaction of T cells with B cells or macrophages (MΦ) can be interpreted similarly^{1,8-15}. We present here evidence that in the mouse, helper T cells primed with the antigen, keyhole limpet haemocyanin (KLH), bound to the surface of MΦ, simultaneously recognise KLH and genetically controlled surface components of the MΦ. The rationale for our approach was as follows. In F₁ animals whose parents differ at loci controlling surface components (for example, H-2, Mls), if T cells could be primed with antigen bound to MΦ from one or the other parent, they should then cooperate preferentially with parental B cells and MΦ of the type used for priming.

To test these predictions we took advantage of the nonspecific association between protein antigens and the MΦ surface¹². As described in the legend to Fig. 1, BDF₁ (C57B/6 × DBA/2), C57BL/6, and DBA/2 mice were injected intraperitoneally with KLH. Peritoneal cells (PC), rich in MΦ were collected and used to prime three groups of BDF₁ mice. T cells isolated from these mice were tested for their ability to cooperate with BDF₁ or C57BL/6 B cells and MΦ in the response to TNP-KLH. All three groups of F₁ T cells were able to cooperate effectively with BDF₁ B-cells and MΦ (Fig. 1a). But as predicted, when tested with C57BL/6 cells and MΦ (Fig. 1b), F₁ T cells primed with KLH bound to C57BL/6 PC were the most efficient helpers, those primed with DBA/2 PC were the least effective and those with BDF₁ PC were intermediate.

Several control experiments have been carried out to eliminate alternative explanations for the results in Fig. 1. For example, it was possible that parental T cells contaminating the PC preparations were stimulated in the F₁ recipients and responsible for the subsequent properties of the primed helper cells. This



For B cells, BDF₁ and C57BL/6 mice were primed with 1.0 µg of TNP-LPS¹⁷. After 7 d spleen cell suspensions were prepared and treated with anti-T-cell serum and complement as described before¹⁸ to deplete T cells but leave macrophages and hapten-primed B cells unaffected. Various combinations of the T-cell and B-cell preparations were tested in the *in vitro* response to TNP-KLH. Cultures (0.5 ml) contained 4×10^6 B cells and macrophages, various numbers of BDF₁ T cells and TNP-KLH (0.05 µg ml⁻¹). After 4 d three identical cultures were pooled and assayed for anti-TNP plaque-forming cells (PFC). Both the culture methods and PFC assay have been described previously^{18,19}. The responses (PFC per culture) are shown compared with the number of T cells added to the cultures. *a*, BDF₁ B cells and MΦ; *b*, C57BL/6 B cells and MΦ. For each titration the response of the B cells and MΦ cultured alone (x) is also shown. These figures represent the average responses obtained in six experiments with similar results.



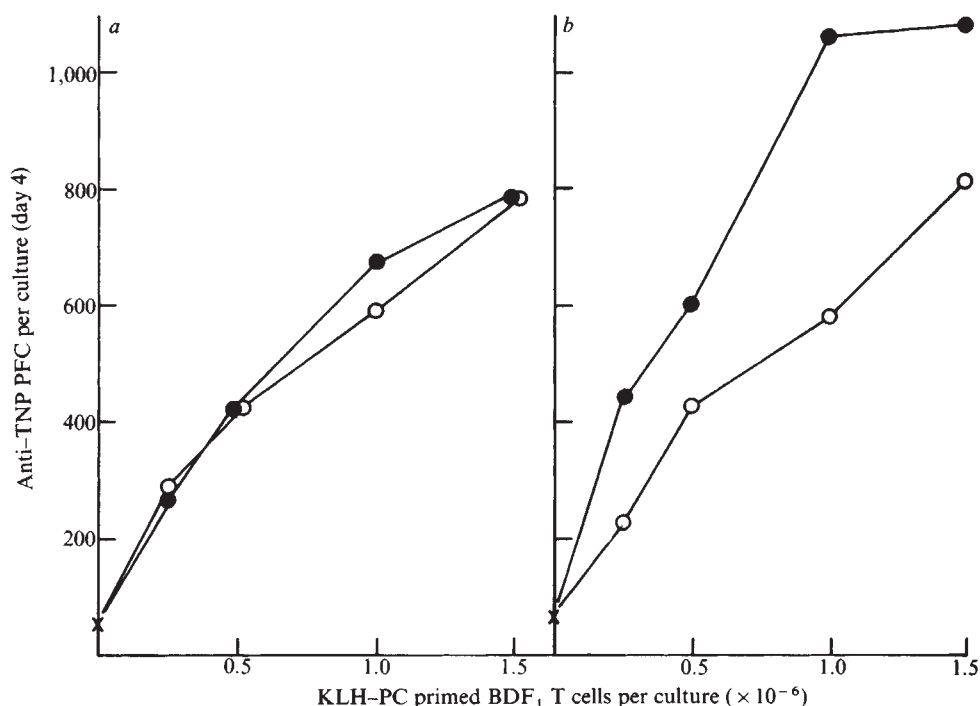
possibility was eliminated by demonstrating that results identical to those in Fig. 1 were obtained even after pretreatment of the KLH-pulsed PC with anti-T-cell serum and complement before priming of F₁ animals (data not shown). In a second control experiment (Fig. 2) we tested whether, rather than a selective priming of helper T cells in the F₁, our results indicated a selective priming of specific suppressor cells. BDF₁ T cells which had been primed with KLH bound to either C57BL/6 or DBA/2 PC were prepared. The helper activities of these T cells and a 1:1 mixture of the two were assayed on C57BL/6 B cells and MΦ. As above, T cells primed with C57BL/6 PC were more efficient than those primed with DBA/2 PC. If the helpers primed with DBA/2 PC were ineffective because of the presence of suppressive activity specific for C57BL/6 cells, then the observed activity of the 1:1 mixture should be less than the calculated average of the responses obtained with the two helper preparations alone. In fact there was no difference between the calculated and observed responses, eliminating a role for a suppressive mechanism in this phenomenon.

We have begun experiments formally to identify the genes expressed in the parental cells that are responsible for imposing this restriction of F₁ helper T cells. For example, BDF₁ mice

Fig. 1 Restriction of F₁ helper T cells in their ability to cooperate with parental B cells and MΦ. Groups of three BDF₁, C57BL/6 and DBA/2 mice (Jackson Laboratory) were injected intraperitoneally with 100 µg of KLH (Calbiochem) in 0.3 ml of balanced salt solution (BSS). After 4 h PC were collected by rinsing the peritoneal cavity with 4 ml of BSS containing 5% foetal bovine serum (FBS). The average yield was 3×10^6 – 4×10^6 PC per mouse. Pooled suspensions of each type of PC were washed twice with 5% FCS in BSS then resuspended to 5×10^6 cells per ml in BSS. Groups of three BDF₁ mice were injected with each type of PC (1.5×10^6 intraperitoneally and 1.5×10^6 intravenously). After 7 d a pooled spleen cell suspension was prepared from each group and depleted of B cells and MΦ on nylon wool columns¹⁶. The resulting T-cell preparations were titrated for KLH-specific helper activity as below. ▲, BDF₁; ●, C57BL/6 and ■, DBA/2 PC-primed BDF₁ T-cells.

Fig. 2 Suppression is not the mechanism of restriction of F₁ T cells. BDF₁ T cells primed with KLH bound to either C57BL/6 (●) or DBA/2 (■) PC were prepared as in Fig. 1. Both T-cell preparations and a 1:1 mixture of the two (▲) were titrated on C57BL/6 B cells and MΦ. The calculated average of the responses seen with each T-cell preparation alone is also shown (---). The response of B cells and MΦ cultured alone is shown (x).

Fig. 3 Involvement of H-2-linked genes in restriction of F_1 T cells. BDF₁ T cells primed with KLH bound to either C57BL/10 (●) or B10.D2 (○) PC were prepared as in Fig. 1. These T cells were titrated on either BDF₁ (a) or C57BL/10 (b) B cells and MΦ as in Fig. 1. In each figure the response of B cells and MΦ cultured alone is shown (×). The figures represent the average responses seen in three similar experiments.



were primed with KLH bound to C57BL/10 (H-2^b) PC or to B10.D2 (H-2^d) PC. The resulting primed helper T cells were titrated for their ability to cooperate with either BDF₁ or C57BL/10 B cells and MΦ. The results (Fig. 3) indicate that although both F_1 T-cell preparations were equally effective when tested on BDF₁ B cells and MΦ, those primed with C57BL/10 PC were more effective with C57BL/10 B cells and MΦ than those primed with B10.D2 PC. Since these two strains are genetically identical except at the MHC, these results suggest a role for H-2-linked genes in the phenomenon. It is interesting that the differences in helper activity seen in Figs 1 and 2, where the priming PC differed at multiple genetic loci were greater than seen in Fig. 3 where the differences were confined to H-2-linked genes. These results may indicate that genes outside the MHC also contribute to the phenomenon.

Several previous studies of the role of histocompatibility gene products on antigen-mediated interactions between T cells and B cells or macrophages have suggested that such interactions require MHC identity between the cooperating cells^{1,8,12}. Other studies^{1,9-11,13,15} and the results presented here enable us to make an alternative interpretation of these findings. They suggest that T cells acquire the restricted ability to cooperate with B cells or macrophages of a particular MHC type, not as a result of some inherent genetic mechanism controlling interactions, but perhaps simply through the selection of relevant clones during the initial contact of T-cell precursors with an antigen in association with the surface components of the MΦ.

We thank Lee Harwell and George Berry for technical assistance. This work was supported by grants from the USPHS and the American Cancer Society.

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Received May 28; accepted July 6, 1976.

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Distribution of β_2 microglobulin and HLA in chorionic villi of human placentae

ANALOGIES are often made between transplantation and placental immunology. The role of histocompatibility antigens is of undisputed importance in transplantation immunology but it is not clear whether such antigens are important in pregnancy. Indeed, it is not known whether trophoblasts manifest histocompatibility antigens. If HLA antigens are relevant in pregnancy, it is of central importance to know if trophoblasts have these antigens. This is a difficult problem because human anti-HLA sera often contain other antibodies, and heterologous anti-HLA sera often lack precise HLA specificity¹. But HLA has been shown to be invariably associated with β_2 -microglobulin², and here we present data in support of the concept that human trophoblasts contain neither β_2 microglobulin nor

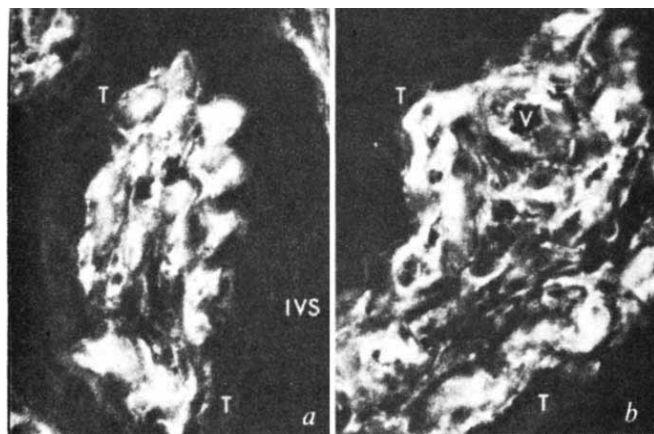


Fig. 1 Immunohistology of full term human placenta with antisera to: *a*, β_2 microglobulin, and *b*, HLA-2. Note negative trophoblasts (T) and positive staining of cells in mesenchymal stroma as well as vessel (V) endothelium. Thin (4- μ m) sections were cut on a cryostat, air dried, and studied by indirect immunofluorescence²³ using an IgG fraction of rabbit antiserum to human β_2 microglobulin (Dakopatts A/S, Denmark) and rabbit anti-HLA sera from Dr A. Sanderson. Anti-HLA sera were prepared to HLA-1, HLA-2, HLA-7 and HLA-12 that were isolated by gel filtration, DEAE-cellulose chromatography and polyacrylamide gel electrophoresis. After absorption of these antisera with human erythrocytes, they were specific at low titre when tested for cytotoxicity with rabbit complement against human peripheral lymphocytes. Fluorescein isothiocyanate (FITC)-labelled sheep anti-rabbit Ig serum was obtained from Wellcome Reagents, Beckenham, Kent. Optimum dilutions of antisera (1:75) and conjugate (1:60) were determined by serial titrations²⁴ that gave minimum nonspecific staining and maximum specificity. Further details are given in the legend of Fig. 3. (Photographed at original magnification of $\times 400$.)

HLA. Also, treatment with several enzymes has failed to reveal these antigens. In addition, we have not been able to identify either β_2 microglobulin or HLA on trophoblasts from 6-week or 13-week placenta or on placental tissues maintained *in vitro* for 5 d.

Thirty-two full term, one 6-week, and one 13-week placenta were studied. All pregnancies were normal and uncomplicated, all neonates were healthy, and all placenta were grossly and microscopically normal. Small pieces of tissue obtained from the central cotyledon within 15 min of delivery were either snap frozen or washed for 16 h at 4 °C with gentle rotation in minimum essential medium supplemented with glutamine, bicarbonate, 10% foetal calf serum (FCS) and antibiotics³, and then snap frozen. *In vitro* cultures were prepared from tissues aseptically dissected

from a 36-week placenta obtained by Caesarian section. Small pieces of tissue were placed on stainless steel grids supported in organ culture dishes (Bio-Cult Laboratories) containing 0.5 ml of medium RPMI supplemented with L-glutamine and 15% FCS (Bio-Cult) and incubated in 5% CO₂ in air. The medium was replaced daily, and the FCS concentration was reduced to 5% after the first day. Cultures were snap frozen after 1, 2 and 5 d in culture.

Tissues that were snap frozen either immediately or after washing overnight in growth medium consistently gave a characteristic pattern of staining for β_2 microglobulin by immunofluorescence. Trophoblasts and trophoblastic basement membranes (TBM) were unstained, and all other cells of the chorionic villus were positive (Fig. 1*a*). Using the antibody-peroxidase technique for light and

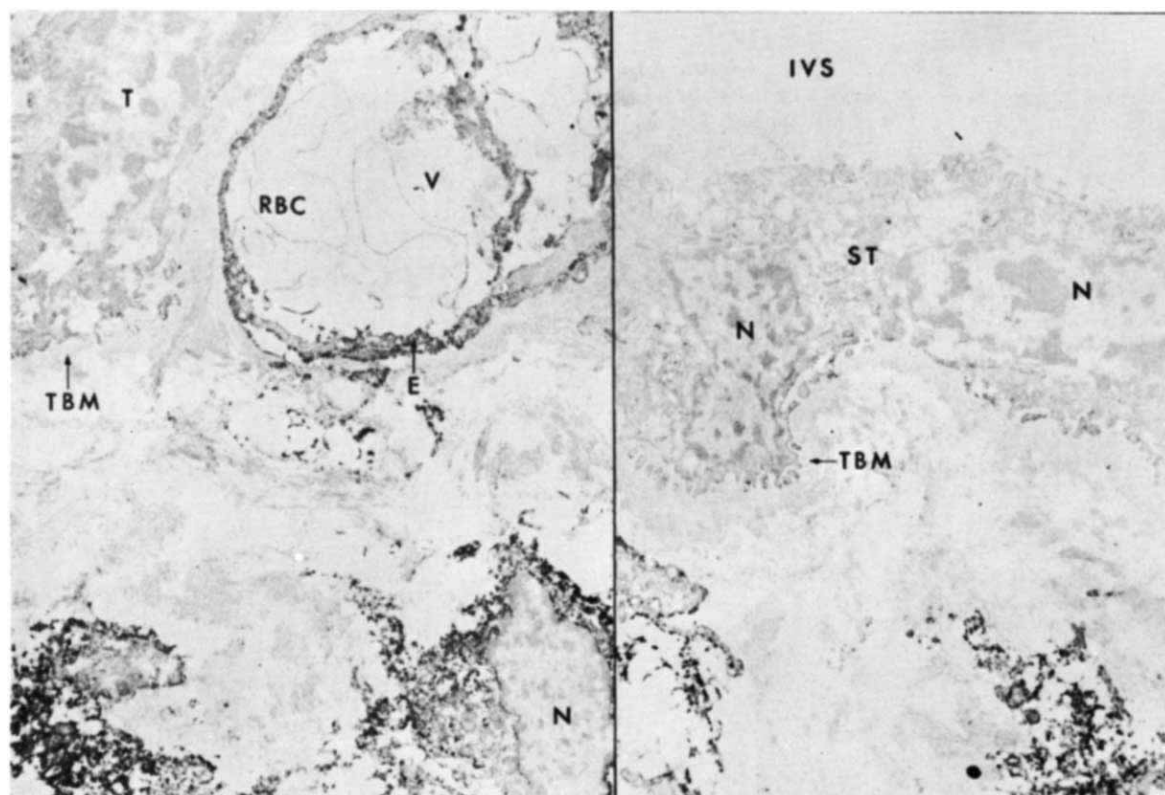


Fig. 2 Immunohistology of full term human placenta with antisera to β_2 microglobulin, achieved using the antibody-peroxidase technique by indirect staining for electron microscopy⁴. An AE1-EM-6B electron microscope was used. IVS, Intervillous space; RBC, red blood cell within vessel (V) in villous stroma; E, vascular endothelium; TBM, trophoblastic basement membrane; N, nuclei of trophoblasts; ST, syncytiotrophoblast. As it is not always possible accurately to differen-

tiate between cytotrophoblasts and syncytiotrophoblasts by immunofluorescence, we restricted morphological definitions of trophoblasts to ultrastructural criteria²⁵. Note negative trophoblasts and TBM, and positive cells and endothelium in villous stroma. Placenta were fixed for 30 min at 4 °C in 1% paraformaldehyde in 0.1 M PO₄, pH 7.4, containing 7.5% sucrose, and washed overnight in 13% sucrose and 0.1 M PO₄. (Original magnification $\times 3,000$.)

Table 1 Effect of enzyme treatment on β_2 -microglobulin staining

Treatment	Dose *	Time of incubation	Temp. (°C)	Trophoblasts	Immunofluorescence Endothelium	Stroma cells
Neuraminidase	3.6	1 h	20	—	++	++
(<i>Clostridium</i>	9.0	2 h	20	—	+	+
<i>perfringens</i>)	9.0	3 h	20	—	+	+
Neuraminidase	500	3 h	20	—	++	++
(<i>Vibrio cholerae</i>)						
Collagenase	920	1 h	20	—	++	++
(Sigma)						
Hyaluronidase	920	1 h	20	—	++	++
(Sigma)	2,300	1 h	20	—	—	++
	4,600	1 h	37	—	—	—
Hyaluronidase	1,500	1 h	20	—	—	++
(Fisons)						
Trypsin	67	30 min	20	—	++	++
(Sigma)	67	1 h	20	—	+	+
PBS	—	2 h	20	—	+	+
	—	18 h	20	—	—	+

Collagenase was dissolved in 0.5 M Tris buffer, pH 7.5, containing 0.05 M Ca^{2+} , hyaluronidase in phosphate-buffered saline (PBS), pH 7.2 and trypsin in PBS, pH 7.4. Neuraminidase from *Cl. perfringens* was dissolved in PBS, and neuraminidase from *V. cholerae* (Koch-Light) was in 0.05 M acetate buffer, pH 5.5 containing 1% NaCl and 1% CaCl_2 .

*Enzyme units ml^{-1} .

electron microscopy⁴, it was possible positively to identify cells staining within villi, and confirm the absence of staining of trophoblasts, TBM and stromal connective tissues (Fig. 2). Tissues from 6-week and 13-week placentae as well as sections from a 36-week placenta that had been cultured *in vitro* for 5 d gave no trophoblast or TBM staining for β_2 microglobulin, but all cells on the foetal side of TBM in these tissues were positive (Fig. 3a and c). Treatment of cryostat sections of the above tissues with neuraminidase, hyaluronidase, collagenase or trypsin also failed to reveal β_2 microglobulin on trophoblasts or TBM by immunofluorescence (Table 1), although exposure to large amounts of hyaluronidase and prolonged washing in phosphate-buffered saline diminished staining reactions of stromal and endothelial cells in some experiments (Table 1).

Several rabbit anti-HLA sera gave a pattern of staining by immunofluorescence identical to that obtained using anti- β_2 -microglobulin serum. No positive staining of either trophoblasts or TBM was obtained with any of the anti-HLA sera, but cells in the villous stroma of all placentae being studied were stained with all these antisera (Figs 1b and 3b). The presence of HLA on endothelial cells of placental vessels is consistent with the report⁵ of HLA on endothelial cells of the umbilical cord. Furthermore, our failure to identify HLA on trophoblasts is compatible with the observation⁶ that human trophoblasts in cryostat sections do not bind concanavalin A. It is interesting that HLA is a glycoprotein that can be partially characterised by its ability to bind to concanavalin A-linked columns⁷.

Whether or not trophoblasts manifest histocompatibility antigens is controversial. For example, Currie *et al.*⁸ have shown that neuraminidase-treated A₂G ectoplacental cone cells injected into CBA mice cause the recipients subsequently to reject donor homografts in an accelerated fashion. Simmons *et al.*⁹, however, using a similar experimental model were unable to confirm this, and our results also do not support the concept of masked histocompatibility antigens being present on human trophoblasts (Table 1). Loke *et al.*¹⁰, using mixed cell agglutination reported that HLA antigens were present on trophoblasts, and Lawler *et al.*¹¹ favour an interpretation of HLA antigens on trophoblasts to explain their results of raised anti-HLA activity of paternal specificity in the sera of women with persistent trophoblastic disease after a first pregnancy with a hydatidiform mole. Perhaps mole tissues do contain HLA, but our experience with a large number of normal placentae suggests that HLA is absent from trophoblasts. The absence of HLA from trophoblasts is consistent with the results of Seigler and Metzgar¹² who used agglutination

and antibody absorption techniques. Faulk *et al.*¹³ have also reported that eluates prepared from exhaustively washed human placentae contain IgG that inhibits mixed leukocyte culture reactions without demonstrating HLA specificity, again suggesting an absence of HLA antigens from trophoblasts.

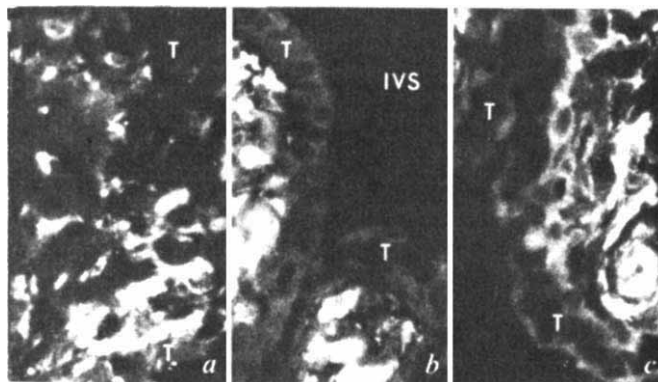


Fig. 3 Immunohistology of: a, 6-week placenta; b, 13-week placenta; c, 5-d culture of term placenta studied with antisera to: a, β_2 microglobulin; b, HLA-7; c, β_2 microglobulin. Specificity and nonspecific-staining controls were done using immunoelectrophoresis, membrane immunofluorescence³ with human leukocytes and red blood cells, and tissue immunofluorescence²³ using human spleen and rat kidney, liver, muscle and stomach as substrates. The anti- β_2 -microglobulin serum gave one line in immunoelectrophoresis against concentrated urine from an oligouric patient with renal amyloid and was negative against five control urines of equivalent concentration. The anti- β_2 microglobulin and the anti-HLA sera gave bright membrane fluorescence at 1:30 dilution with human leukocytes but failed to stain human red cells. These antisera also stained leukocytes in human spleen but did not stain any of the rat tissues. Normal rabbit, sheep, and human sera were used as controls in all experiments. Sera were absorbed for 30 min at 18 °C before use by gentle rotation with an equal volume of washed, packed, sheep erythrocytes, and staining reactions were done according to standard methods^{23,24}. Tissues prepared for immunofluorescence were examined by transmitted light using a Reichert Zetopan microscope fitted with a quartz-iodine light source, a Tiyoda wide-field dark-ground condenser incorporating a toric lens, and a FITC-3 Balzer interference primary filter and a Wratten 12 barrier filter. Photographs of transmitted fluorescence were made using Agfa-Gevaert CT18 (ASA 50) 35-mm film. Tissues were also examined by incident light using a Zeiss Photomicroscope 11 fitted with a Ploem epi-illuminator and an HBO 200 high-pressure mercury-vapour burner light source. Photographs of incident light were made using Gaf 500 (ASA 500) high speed 35-mm film. IVS signifies intervillous space, and correspondingly black areas on other photographs are also IVS. (Photographed at original magnification of $\times 400$.)

Heyner¹⁴, using direct immunofluorescence has reported H-2 antigens on cells of 6.5-d embryo cells but not on trophoblasts, and Searle *et al.*¹⁵, using cytotoxicity reactions and a tailskin homograft model, found no evidence for histocompatibility antigens on trophoblasts, but did find H-2 antigens on cells of the embryonic sac. Billington¹⁶ and Gardner *et al.*¹⁷ have also questioned the presence of histocompatibility antigens on mouse trophoblasts, and Searle *et al.*¹⁸, using the antibody-peroxidase technique for electron microscopy, have shown H-2 antigens on blastocysts which are lost from the trophoblast at the time of implantation.

Experimental models that necessitate the physical separation of trophoblasts from other placental cells may contaminate trophoblast preparations with cells of the villous stroma that bear histocompatibility antigens. Similar technical considerations could account for the diversity of opinion regarding the presence of HLA or H-2 on trophoblasts. The geometrical relationship of trophoblasts to TBM and stroma is retained on cryostat sections studied by immunofluorescence, and using this approach we have been able to show a type of antigenic segregation in the distribution of β_2 -microglobulin and HLA within chorionic villi. It is also important to know, however, whether or not histocompatibility antigens are present on the cell membranes of trophoblasts. This could technically be done by membrane fluorescence³, using trophoblasts from *in vitro* cultures, but the possibility of contaminating trophoblast preparations with other villous cells still exists. What is needed is a membrane marker for human trophoblasts, and preliminary studies suggest that this might be transferrin¹⁹.

These observations of the absence of histocompatibility antigens on trophoblasts suggest that analogies between transplantation and placental immunology are not entirely correct. There is no apparent mechanism to explain why trophoblasts lack β_2 -microglobulin and HLA when these antigens are present on cells within mesenchymal stroma of the same organ. Genetic studies have shown, however, that the absence of β_2 -microglobulin and HLA is not unique to trophoblasts. The B-cell-derived human Daudi lymphoblastoid line lacks both these proteins, but retains the capacity to express products of immune response-associated genes²⁰. Goodfellow *et al.*²¹ have shown that the gene responsible for β_2 microglobulin is not in the HLA region but is on chromosome 15, and Heyningen *et al.*²² have reported that the genes responsible for mannose-phosphate isomerase and pyruvate kinase are also located on chromosome 15. We are studying the ability of human trophoblasts to manifest these enzymes. If the absence of β_2 microglobulin and HLA is associated with the specific capacity of human trophoblasts to avoid maternal recognition, then an understanding of the mechanism will perhaps be useful as an approach to biomedical and pharmacological studies of the more general problem of escape from immunological surveillance.

We thank Dr A. Sanderson for anti-HLA sera and information about their preparation and specificity; Dr Nancy Smith for 6-week and 13-week placentae; Drs Peter Johnson and Peter Page-Thomas for useful discussions; Mr Jack Dorling for assistance with antibody-peroxidase experiments; Mr Robin Faulk for technical assistance, and the WHO and the MRC for support.

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Human milk samples from different ethnic groups contain RNase that inhibits, and plasma membrane that stimulates, reverse transcription

WE have compared human milk samples from American, Hindu and Parsi donors with a view to determining the relative amounts of reverse transcriptase inhibitors¹⁻⁵ present in them. Our intention was to find out whether samples from any specific ethnic group are advantageous for the study of type B virus-like particles in milk^{2,6-8} and for purification of virus-associated enzymes. We also hoped that when sufficient data become available, such studies would be interesting from an epidemiological point of view, to test whether the inhibitors provide protection from mammary cancer. We have confirmed the presence of RNase in milk samples, and have observed variations in the level of RNase in samples from different ethnic groups. Careful examination has also revealed the presence of a protein factor, associated with plasma membranes in milk samples, that stimulates RNA-directed DNA synthesis (RDDS) *in vitro* in oncornaviruses.

To determine the effect of RNase-like inhibitors in different milk plasmas, each was examined for RNase content as judged by its ability to degrade ¹⁴C-labelled yeast RNA (Table 1). The percentages shown against each group are averages of 15 independent experiments. Each sample tested for RNase activity was also tested for possible inhibitory activity in RDDS (see legend to Table 1). The mean values obtained are listed in Table 1 against the different ethnic groups. Although the mean values differ, the standard deviations are large and so it is difficult to place too much emphasis on the differences at this stage. With a view to minimising deviations arising from various factors involved in the enzyme assays, a more direct quantification was attempted.

Figure 1a, b and c shows the results on the basis of quantitative correlation for individual samples. In each graph all experimental points derive from a single set of experiments. The RNase-resistant counts for the individual samples are plotted on the abscissa, and the corresponding ³H-TTP counts incorporated in the reverse transcriptase reaction using AMV pelleted through the milk samples are plotted directly on the ordinate. In a single set of experiments the same reaction mixture, with identical specific activity of ³H-TTP, was used

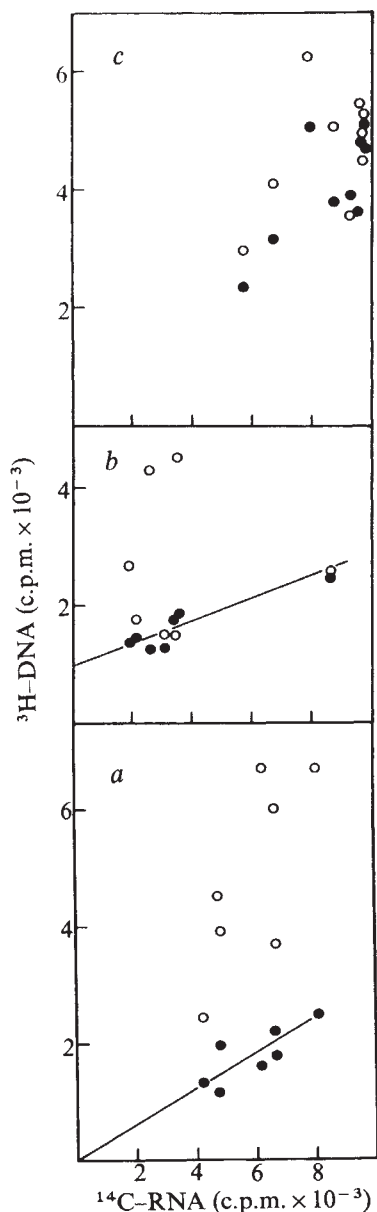


Fig. 1 Direct correlation of RNase content in milk samples and ^3H -TTP incorporation in reverse transcriptase reaction using AMV pelleted through the milk samples. Data from a single set of experiments from the different ethnic groups are presented. Milk plasma (2 ml) from each sample was halved. One half was used for incubation with ^{14}C -labelled yeast RNA, and the other for mixing with AMV (see legend to Table 1 and text). The AMV recovered after pelleting was halved, one half was used for primary reaction using endogenous template, and the other for the reaction with synthetic template. RNase-resistant counts are plotted on the abscissa and the ^3H -TTP incorporation in the parallel reverse transcriptase reactions plotted on the ordinate (see text). ●, Primary reactions; ○, reactions on synthetic template. *a*, American samples; *b*, Hindu samples; and *c*, Parsi samples. Specific activity of ^3H -TTP in primary reactions: 2,500 c.p.m. pmol^{-1} ; 800 c.p.m. pmol^{-1} with synthetic template.

and the numbers obtained for ^3H -TTP incorporation were used directly to test possible correlation with the RNase-resistant counts. This provided a more direct comparison than plotting percentage RNase-resistance against percentage incorporation of label.

For samples from American and Hindu donors there was a direct correlation between RNase-resistant RNA counts in the milk plasma and DNA synthesis in primary reactions. This was not so for poly(dT) synthesis with poly(dT)·poly(rA) as template: which is understandable as the susceptibility to

RNase of a single-stranded viral RNA template would be expected to be different from that of a synthetic hybrid template. Figure 1c shows that for the Parsi samples most points are grouped together towards the extreme right hand region of the curve. Very few points reach even towards the central region. Together with the data in Table 1 for the mean RNase level, these observations indicate a lower level of RNase in Parsi samples.

Although most milk samples contained inhibitors of reverse transcriptase, 40% of the Parsi samples did not inhibit RDDS. And in three out of 15 samples (Table 1) there was enhanced DNA synthesis compared with the control. Figure 2a shows the kinetics of an AMV reaction in which enhancement was observed. Samples belonging to this group have been tested repeatedly and the observation has been validated both for AMV and murine mammary tumour virus. Similar behaviour was observed for two of the 15 American samples, and later in several more Parsi samples (unpublished results). The stimulation observed in all these cases was RNase sensitive.

In a variation of this experiment the milk pellet prepared without AMV was taken up in 0.01 M Tris (pH 8.3), and then mixed with AMV instead of pelleting AMV through milk plasma. This had no effect on the observed results. If the milk pellets alone from these samples were tested for reverse transcriptase, there was no detectable reaction which could

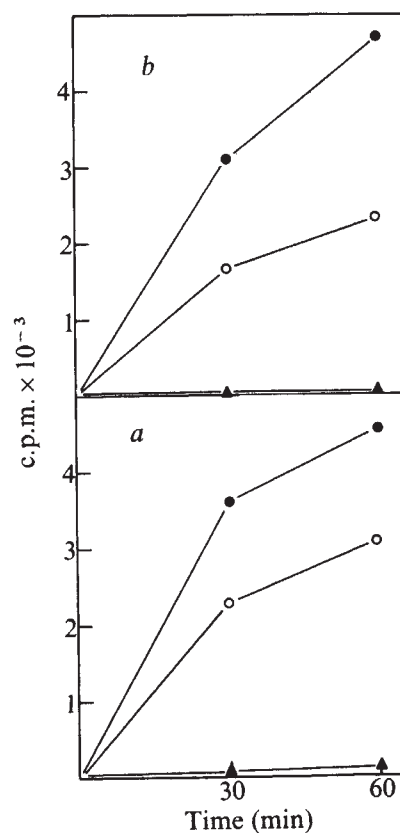


Fig. 2 Kinetics of stimulation of AMV primary reactions by plasma membrane factor from milk pellet (*a*, see text) and from milk fat suspension (*b*). ▲, Control reaction without AMV, but milk pellet alone (*a*) or fat suspension (*b*). ○, AMV primary reaction without any added milk component. ●, AMV primary reaction with milk pellet (*a*) corresponding to 60 μg of total protein, and with fat globule suspension (*b*) corresponding to 200 μg of fat with 10 μg of membrane-associated proteins as determined by Lowry method. In either case the milk factor was added to AMV before incubation with Nonidet P-40. The stock fat globule preparation was made by suspending freshly separated fat from milk plasma in 0.01 M Tris, pH 8.3, at 37 °C at a concentration of 20 mg ml^{-1} , and mixing thoroughly in a vortex mixer before pipetting out the required amount.

Table 1 Inhibition of RDDS by human milk plasma

Ethnic group	mg fat per ml	% RNase-resistant counts*	% DNA synthesis in AMV pelleted through milk plasma†	
			Primary	Synthetic template
American	9.9±2.5	57.2±23.8	78.7±17.1	87.2±15.9
Hindu	17.7±7.8	62.1±14.3	63.3±18.5	76.1±14.3
Parsi	22.2±2.8	81.3±19.6	89.9±10.3	95.1±11.2

Hindu and Parsi milk samples were collected from maternity homes in Bombay and the samples were cooled immediately in ice, and later stored in liquid N₂. American samples were from the Michigan Cancer Foundation. The milk plasma was separated and the viral pellet prepared by the method of McCormick *et al.*⁵. The fat layer separated from a known amount of milk sample was extracted twice with a mixture of chloroform-methanol (2:1), the pooled extract was filtered and the filtrate was evaporated to dryness at 80 °C under a jet of dry nitrogen. The amount of dry residue left was determined by direct weighing and the content of fat in milk (mg ml⁻¹) was calculated. The relative amount of RNase in the samples was determined by incubating known amounts of ¹⁴C-labelled yeast RNA (~8,000 c.p.m.) in 1 ml of the plasma for 30 min at 0 °C and determining the trichloroacetic acid (TCA)-precipitable counts after incubation. The percentage acid precipitable counts after incubation was determined for 15 samples each from the different ethnic groups and the mean values were calculated with standard deviations. Incubation of yeast RNA in control experiments at 0 or at 37 °C made no appreciable difference to the degradation of the RNA, and so we chose 0 °C as the temperature of incubation. Reverse transcriptase reactions were carried out as described before^{8,14}. To determine the inhibition of AMV reaction by milk plasma, AMV (corresponding to 50 µg of viral protein) was mixed with 1 ml of milk plasma and kept at 0 °C for 30 min. This was layered over 4 ml of 20% glycerol in TNE (0.01 M Tris, pH 7.2, 0.1 M NaCl and 0.002 M EDTA) and the virus was pelleted by centrifugation at 234,000g for 30 min in a Beckman 50.1 rotor. The residue was taken up in 0.01 M Tris, pH 8.3, and used for reverse transcriptase reactions. ³H-TTP incorporation from an equal amount of AMV (without milk plasma) pelleted through 20% glycerol was used to determine percentage DNA synthesis. The numbers reported are mean values from 15 determinations for each ethnic group. The specific activity of ³H-TTP used was 2,500 c.p.m. p mol⁻¹ for primary reactions, and 800 c.p.m. p mol⁻¹ for reactions with synthetic template.

* % Acid precipitable ¹⁴C-RNA counts after incubation for 30 min at 0 °C.

† % ³H-TTP incorporation in reverse transcriptase reactions using AMV pelleted through milk plasma compared with AMV pelleted through TNE.

account for the stimulation of AMV RDDS by an equivalent amount of pellet (Fig. 2a). Thus it is unlikely that the stimulation results from milk viral RNA-dependent DNA synthesis. We also examined the possibility that the enhanced synthesis was due to the protection of the AMV reverse transcriptase from proteolytic enzymes, as a consequence of the presence of additional proteins in the milk pellets. In control reactions in which an equivalent amount of either bovine serum albumin or casein was added in place of milk pellets there was no increase in the incorporation of ³H-TTP.

In a separate experiment, a sample of milk pellet treated with Nonidet P-40, that stimulated AMV, was equilibrated with DEAE-cellulose for 30 min at 4 °C. The slurry was poured

on to a column (0.7×3 cm), washed with buffer (0.01 M Tris, pH 8.3, 2 mM dithiothreitol (DTT) and 10% glycerol) containing 0.05 M NaCl, and eluted with the same buffer containing 0.4 M NaCl. RDDS stimulatory activity was present in the eluate. No activity remained either in the 0.05 M NaCl wash or in the loading solution draining out of the column after equilibration with DEAE-cellulose.

We have made similar observations with the fat components. Reports have implicated lipid-associated inhibitor(s) present on milk particle isolates^{1,4,9}, but no systematic investigation of any such inhibitors has been reported. We observed variations in fat contents of milk samples from different ethnic groups (Table 1), and the freshly separated fat portions were tested for the presence of inhibitors. With samples for which the milk pellets showed strong inhibition of AMV reverse transcriptase, the addition of the corresponding fat suspension also showed inhibition. With samples that showed no inhibition, addition of the fat suspension had no inhibitory effect on ³H-TTP incorporation in AMV reactions. There was, on the contrary, increased incorporation of ³H-TTP in about 30% of such samples. This was clearly noticeable in the Parsi samples. Furthermore, samples showing enhanced DNA synthesis with milk pellets in AMV RDDS invariably stimulated the AMV reaction with the fat components as well (Fig. 2b).

We have observed stimulation of DNA synthesis¹⁰ in the AMV reverse transcriptase reaction by plasma membrane preparations from chicken embryonic cells. To test the possibility that the stimulation in AMV reactions caused by milk plasma pellets, as well as fat globules, results from the presence of membranes in the preparations, these fractions were analysed carefully. Electron micrographs indicated the presence of small but definite amounts of membranes present in the plasma pellet preparations in the conditions⁵⁻⁷ in which these pellets are made for detecting RDDS. In addition, both the pellets and fat fractions were layered on discontinuous sucrose gradients to enrich the membrane preparations which were assayed for marker enzymes (Table 2): the same preparations in turn stimulated AMV RDDS. Even when a milk pellet or fat fraction was not stimulatory at first, once the membranes were purified they were stimulatory. There is evidence that the fat globules are covered by a double layer membrane which is very similar in composition to plasma membrane¹¹⁻¹³, suggesting that the stimulation from the milk pellet and from the fat component results from the same membrane-bound factor. This factor, like that isolated from chicken embryos¹⁰, is not dialysable and yet is inactivated by heat. Experiments to enrich

Table 2 Membrane marker enzymes in human milk samples

Marker enzyme	Specific activity of enzyme per mg protein		
	Casein pellet	Ultracentrifuge pellet from milk plasma	Fat fraction
Alkaline phosphatase	0.326 µmol	0.283 µmol	0.107 µmol
Acid phosphatase	0.360 µmol	0.200 µmol	0.890 µmol
Xanthine oxidase	236 U	194 U	178 U

Membrane marker enzymes (selected from those listed in ref. 11) were assayed from different fractions of the milk samples. The casein pellet was obtained by centrifuging the milk sample at 5,000 r.p.m. in a Sorvall HB4 rotor for 20 min. The fat fraction is obtained by removing the top layer of fat, and the milk plasma pellet was obtained by layering the intermediate layer of milk plasma on 20% glycerol (see legend to Table 1) and isolating the pellet obtained by centrifugation at 234,000g for 30 min in a Beckman 50.1 rotor. To enrich the membranes, these fractions were suspended separately and uniformly in TM buffer (0.05 M Tris, pH 7.4, and 0.005 M MgCl₂) containing 20% sucrose. The fat suspension was treated differently. Skimmed fat (1 ml) was suspended in 5 ml of TM buffer and homogenised in a Potter-Elvehjem homogeniser (30 strokes). This was centrifuged at 10,000 r.p.m. in an HB4 rotor for 10 min and the pellet was collected. The fat layer remaining at the top was collected, rehomogenised and pelleted in the same way twice more. The pellets were pooled and suspended in TM buffer containing 20% sucrose. The different suspensions in TM containing 20% sucrose were layered over a discontinuous sucrose gradient (TM) with 60% sucrose in the bottom overlain with 50, 40, 35 and 30% sucrose solutions. The samples were centrifuged at 1,380g in an HB4 rotor for 15 min. The band between 35 and 40% sucrose was collected. The membrane was isolated by repelleting after dilution with TM. This was dissolved in appropriate buffers for different enzyme assays. Alkaline phosphatase was assayed as described by Malamy and Horecker¹⁵, acid phosphatase as described by Hollander¹⁶ and xanthine oxidase by the method of Roussos¹⁷. Incubation was for 1 h at 37 °C.

the stimulatory factor as well as to characterise the stimulated DNA product are in progress.

We thank Professor J. Beard for AMV.

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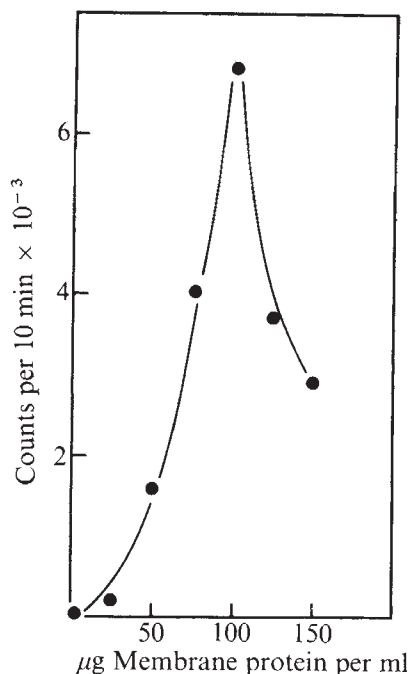
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Role of plasma membranes in stimulation of RNA-directed DNA synthesis

THERE has been considerable speculation about the small size of DNA transcripts (less than 10S) obtained by reverse transcription *in vitro* of 60-70S RNA from oncornaviruses¹⁻⁴. On the basis of evidence suggesting that at least part of the virus-specific DNA synthesis could be taking place at the plasma membrane^{5,6}, we have examined the role of membrane-bound factors on RNA-directed DNA



synthesis (RDDS). We report here the existence of a protein factor, associated with plasma membrane preparations from chick embryonic cells, that stimulates RDDS in avian myeloblastosis virus (AMV). The stimulated synthesis is sensitive to RNase, and the product DNA is 33-35S in alkaline sucrose gradients.

The effect of added plasma membrane on AMV incubated with Nonidet P-40 is shown in Table 1. The properties of the purified membrane preparations are shown in Table 2. There was a marked increase in ³H-TTP incorporation in the primary reaction with AMV endogenous template. Similarly, there was increased incorporation of ³H-dGTP when the synthetic template oligo(dG)-poly(rC) was used. In the absence of AMV there was no DNA synthesis with membrane preparation alone, or in conjunction with oligo(dG)-poly(rC). This rules out the possibility that any DNA-polymerising enzymes were present in the membrane and contributed to the increased synthesis.

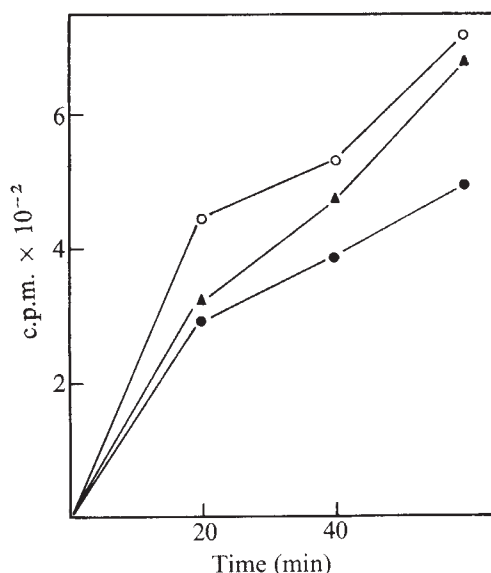


Fig. 2 Solubilisation of stimulation factor. Plasma membrane preparation containing 1 mg of proteins per ml was made 0.4% with respect to Nonidet P-40 and 0.1 M with respect to DTT. The mixture was incubated in ice for 30 min and was centrifuged in a Sorvall HB4 rotor at 7,500 r.p.m. for 10 min. The supernatant was removed and the pellet was resuspended in 0.01 M Tris, pH 8.3. After determining the protein concentrations in the supernatant and the pellet suspension, equivalent amounts of protein were added to AMV reverse transcriptase incubation mixtures (see legend to Table 1). From reaction mixtures containing 100 µg of membrane protein per ml, 30-µl samples were withdrawn at different times and the acid-precipitable radioactivity determined. ○, Supernatant; ▲, whole; ●, pellet.

Figure 1 shows the dependence of stimulation on the concentration of the membrane preparation in the reaction mixture. We found that in our experimental conditions the optimum concentration corresponded to 100 µg of membrane proteins per ml of reaction mixture. It was similar when a synthetic template was used. The reason for this is unknown. It is likely that there is a stimulatory activity and an inhibitory factor present in the unfractionated membrane proteins. Even after the stimulatory activity

Fig. 1 Stimulation of RNA-directed DNA synthesis by AMV in presence of increasing amounts of plasma membrane. The results of primary reactions with endogenous RNA template are shown. Reactions were carried out in conditions described in the legend to Table 1. Incorporation of ³H-TTP into TCA-precipitable radioactivity in 45-min reactions was determined, as were excess counts above control (reaction with no membrane protein present) and are plotted against different membrane concentrations.

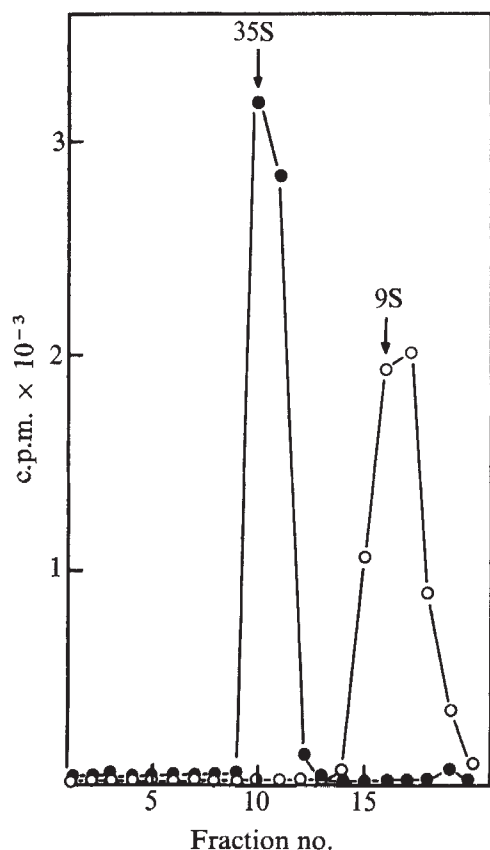
Table 1 Stimulation of AMV reverse transcription by plasma membrane preparations

Reaction	c.p.m. of ^3H -TTP incorporated in 40 min
Primary reaction	
AMV control reaction	3,200
AMV + plasma membrane (120 μg protein per ml)	6,024
Membrane without AMV (120 μg protein per ml)	36
Reaction using oligo(dG)·poly(rC)	
AMV control reaction	6,172
AMV + plasma membrane (120 μg protein per ml)	21,399
Membrane without AMV (120 μg protein per ml)	81

Plasma membrane was prepared from 7–9-d-old chicken embryos as described in the legend to Table 2. The amount of total protein present in each preparation was determined by the Lowry procedure and purity was checked by marker enzymes (Table 2). The standard reverse transcriptase assay mixture contained, 5×10^{-2} M Tris, pH 8.3, 4×10^{-2} M NaCl, 8×10^{-3} M MgCl_2 , 2×10^{-3} M dATP, dCTP and dGTP, and ^3H -TTP at 1,500 c.p.m. pmol^{-1} for the primary reactions. The mixture for the synthetic template reactions contained oligo(dG)₁₂₋₁₈·poly(rC) at a concentration of $10^3 \mu\text{g ml}^{-1}$. Of the triphosphates these mixtures contained only cold dCTP (2×10^{-3} M) and ^3H -dGTP (500 c.p.m. pmol^{-1}). The membrane preparations (1 mg ml^{-1}) were made 0.4% with respect to Nonidet P-40 and 0.1 M with respect to dithiothreitol (DTT) and incubated separately at 0°C for 30 min. Required amounts of this preparation were added to the standard reverse transcriptase assay mixtures before incubation at 37°C . The primary reactions were in the presence of actinomycin D ($50 \mu\text{g ml}^{-1}$). The reactions described above were done using 50 μg of AMV preincubated for 10 min at 0°C with 0.4% Nonidet P-40 and 0.1 M DTT. The total reaction volume was 100 μl . Samples of 30 μl were withdrawn after 0 min, 20 min and 40 min of incubation and the trichloroacetic acid (TCA) precipitable radioactivity present in the different samples was determined.

reaches saturation, the inhibitory activity may be increasing as membrane concentration increases.

The stimulatory activity associated with the membrane preparations can be solubilised. When a preparation was centrifuged after incubation with Nonidet P-40 the activity



was present in the supernatant. But, not all the activity was recovered (Fig. 2); some remained in the pellet fraction, although at a reduced level. In a different experiment, the membrane preparation from chick embryo cells, after treatment with Nonidet P-40, was centrifuged in a Sorvall HB 4 rotor at 7,500 r.p.m. for 10 min, and the supernatant was equilibrated with DEAE-cellulose for 30 min at 4°C . The thick slurry was poured on to a column ($0.7 \times 2 \text{ cm}$), washed with buffer (0.01 M Tris, pH 8.3, 2 mM DTT, 10% glycerol) containing 0.05 M NaCl, and eluted with the same buffer containing 0.4 M NaCl. RDDS stimulatory activity was present in the eluate, and none remained in either the 0.05 M NaCl wash or the loading solution draining out of the column. The activity was destroyed by heating for 10 min at 100°C and it was non-dialysable. These observations, together with the fact that stimulation was observed when a synthetic template was used with only two deoxynucleoside triphosphates in the reaction mixture, eliminate

Fig. 3 Comparison of the size of DNA product of AMV reverse transcription in the presence and absence of plasma membrane preparations. The DNA products were made using reaction conditions described in the legend to Table 1. For size determination we have used reaction mixtures containing ^3H -TTP at 2,500 c.p.m. pmol^{-1} . After incubation for 60 min the reactions were stopped by the addition of sodium dodecyl sulphate to 1% and NaCl solution to 0.4%. The nucleic acid products were isolated by phenol extraction as described before¹⁶. The isolate was incubated with 0.3 M NaOH for 18 min at 37°C and after cooling was layered on a 5–20% linear sucrose gradient (10^{-2} M Tris; 10^{-2} M EDTA; 0.7 M NaCl, and 0.3 M NaOH). Centrifugation was done in a Spinco SW50.1 rotor at 48,000 r.p.m. for 120 min. The gradients were fractionated and the TCA-precipitable radioactivity was determined. Purified DNA from phage PIKC, and *Haemophilus influenzae* phage (HPIC₁) were used as external markers in separate tubes, ●, DNA synthesised in the presence of plasma membrane (110 μg proteins per ml); ○, DNA synthesised in control reactions without added membrane proteins.

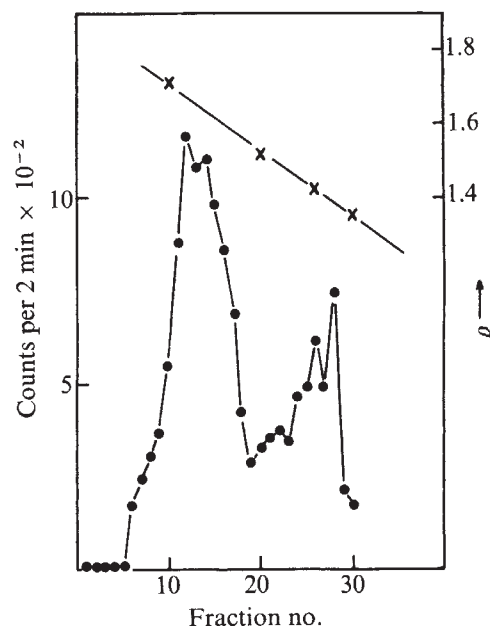


Fig. 4 Hybridisation of DNA product from a plasma membrane stimulated AMV primary reaction with high molecular weight AMV RNA. Purification of single-stranded DNA product and preparation of AMV high molecular weight RNA have been described before¹⁶. The incubation mixture for hybridisation contained, 50 μg of AMV RNA; 10,000 c.p.m. of purified ^3H -DNA; 0.45 M NaCl, and 0.002 M EDTA, pH 7.2 and 75 μl formamide in 150 μl . After incubation at 37°C for 24 h, the solution was diluted with 2.35 ml of 5×10^{-2} M EDTA and 2.56 ml of saturated Cs_2SO_4 . This solution had a refractive index of 1.375. It was centrifuged for 60 h at 20°C using a Spinco SW50.1 rotor at 40,000 r.p.m. After centrifugation the contents were fractionated by bottom puncture of the Polyallomer centrifuge tube and the TCA-precipitable radioactivity in the fractions determined.

Table 2 Marker enzymes present in plasma membranes isolated from chick embryos

Enzyme	Specific activity per mg per h		
	Homogenate*	Purified preparation	Enrichment factor
ADPase	1.02 μ mol	2.31 μ mol	2.3
5'-Nucleotidase	0.49 μ mol	2.00 μ mol	4.1
Na ⁺ , K ⁺ -ATPase	0.75 μ mol	2.00 μ mol	2.7
Alkaline phosphatase	0.16 μ mol	1.40 μ mol	8.8
CTPase	0.82 μ mol	4.04 μ mol	4.9
Succinate dehydrogenase	8.7 U†	2.1 U†	0.24

*Homogenate after filtration through cheesecloth (see below)

†Increase in A_{490} units; see ref. 15.

The membranes were made from 7-9-d-old chick embryos^{8,9}, which were dissected to remove internal organs, head and limbs. The tissue was minced, washed thoroughly with TM buffer (5×10^{-2} M Tris, pH 7.4 and 5×10^{-3} M $MgCl_2$) and homogenised by 30 gentle strokes in a Potter-Elvehjem homogeniser. It was filtered through two layers of cheesecloth. It was centrifuged at 2,500 r.p.m. in a GSA rotor for 15 min and the pellet was resuspended in TM and placed on top of a discontinuous sucrose gradient in TM consisting of 60% sucrose in the bottom (4 ml) overlain with solutions containing 50% (4 ml), 40% (4 ml), 35% (10 ml), 30% (5 ml) and 20% (5 ml) of sucrose. The gradient was made in a cellulose nitrate tube for the Beckman SW27 rotor, but spun in an HB4 rotor at 1,380g for 15 min. The fraction that collected between 35% and 40% sucrose was withdrawn with a syringe and pelleted by centrifugation at 6,000g for 15 min after adjusting the sucrose concentration to 5%. This pellet was resuspended in TM and purified twice more by passage through the discontinuous gradient and isolation between 35% and 40% sucrose. The pellet obtained after the third discontinuous gradient was taken up in 30% sucrose in TM and placed over a different discontinuous gradient with 60% sucrose at the bottom (3 ml) overlain with solutions having 55% (3 ml), 50% (3 ml), 48% (6 ml), 45% (6 ml) 43% (4 ml), and 40% (4 ml) of sucrose and centrifuged at 90,000g for 90 min in a SW27 rotor. The membrane formed a band between 43% and 40% sucrose. This fraction was separated and the membrane pelleted by centrifugation at 6,000g for 15 min after adjusting the sucrose concentration to 5%. This was resuspended in the required buffers for the assay of marker enzymes. The amount of total proteins present in each preparation was determined by the Lowry procedure. ADPase and CTPase were assayed as described by Perdue and Snider¹⁰, Na⁺, K⁺-ATPase by the method of Post and Sen¹¹, 5'-Nucleotidase by the method of Morre¹², alkaline phosphate by the method of Malamy and Horecker¹³, and succinate dehydrogenase by methods described by King¹⁴ and Pennington¹⁵.

the possibility that stimulation was due to a DNA or RNA contaminant in the membrane preparation.

The DNA product of the stimulated primary reactions was larger (33-35S in alkaline sucrose gradients (Fig. 3)), than the product synthesised in the absence of plasma membrane preparation. The distribution of size varied slightly from experiment to experiment: this could have resulted from variations in length of viral RNA template.

The hybridisability of the product was also examined. The results of hybridisation with 70S RNA from AMV are shown in Fig. 4. Most of the counts were present in the RNA and RNA-DNA hybrid density region. The spilling over of the counts to the hybrid region when hybridisation was done with an excess of AMV RNA probably indicates the presence of full length DNA transcripts. Unlike small pieces of DNA hybridising to large RNA molecules, resulting in RNA-DNA hybrids with densities close to that of RNA, full length DNA or nearly full length DNA can form 1:1 RNA-DNA hybrids. This type of hybrid would band at a region with the mean density of DNA and RNA.

Based on these observations, we suggest that the stimulation of DNA synthesis is caused by a thermosensitive and non-dialysable factor associated with the membrane. But the method used to purify the membrane may also have purified some of the membranous organelles of the cell, such as endoplasmic reticulum, for their density is very close to that of the outer plasma membrane. At present the mechanism of stimulation and chain elongation is not clearly understood. Experiments are in progress to determine

whether the stimulation of the reaction is due to interaction of the factor with the template or reverse transcriptase. In this respect, the factor can be compared with the DNA binding proteins reported by Hung and Lee⁷. Rigorous characterisation (such as genomic complexity) of the longer DNA molecules synthesised in the presence of the membrane preparation is in progress. If the longer DNA molecule contains the same information as the viral RNA faithfully represented in the same proportion, such radioactive molecules will provide 'superior probes' in studies of viral gene expression in infected cells.

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Received April 2; accepted July 5, 1976.

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Ion effects on macromolecules in aqueous solution

APART from their nonspecific electrostatic interactions, ions can produce specific effects on the conformation and solubility of macromolecules in aqueous solution¹. The relative effectiveness of anions and cations in altering macromolecular configurations is remarkably independent of the nature of the macromolecule and is generally referred to as the lyotropic or Hofmeister series. To explain the generality of the effects, the influence of the ions on the structure of the solvent has often been invoked, but as yet no theory has been able to link satisfactorily ion-water interactions with the effects of ions on macromolecules. We argue here that it is the effect of the ions on the empty volume of the solvent which underlies the Hofmeister series and we derive a simple thermodynamic equation which accounts for the effect of a salt on the transition temperature for a polymer chain aggregation process. To test the equation we have taken data from the literature relating to the cloud point of polyvinyl-methylether (PVME; intermolecular aggregation); and to the thermal transition temperature of ribonuclease (intramolecular aggregation).

Aqueous solutions of PVME have a lower critical solution temperature or cloud point (T_c) at 34 °C—that is, above this temperature two immiscible phases are formed, one of which contains almost all the polymer in an aggregated form. On cooling the change is reversed but redissolution of the polymer is slow. Horne *et al.*² studied the effect of a number of salts on T_c and found that it could be either raised or lowered according

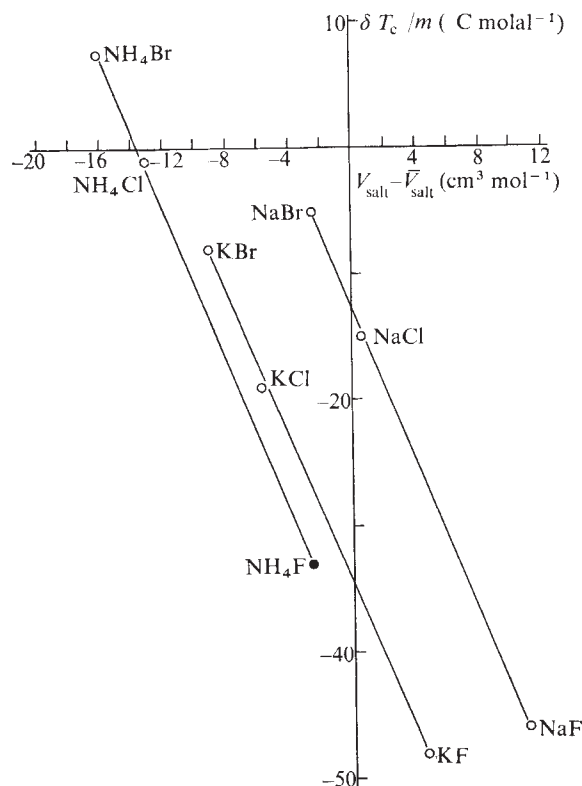


Fig. 1 Salt effects on T_c of a 2.5 weight % aqueous solution of PVME. $\circ$, Ref. 2; $\bullet$, present work.

to the particular salt added and that often the change was approximately linear with salt concentration. Similarly, it has been shown that the temperature (T_m) of the midpoint of a cooperative transition involving the unfolding of the native ribonuclease molecule can be shifted up or down, often approximately linearly by increasing concentrations of neutral salts^{3,4}. In both cases the rank order of molar effectiveness follows the Hofmeister series.

At either T_c or T_m we can write an equilibrium: dissolved polymer segments $\rightleftharpoons$ aggregated polymer segments. The transfer from the dissolved to the aggregated state in water at a pressure P will involve changes in the partial molal internal energy ($\bar{U}$), volume ($\bar{V}$) and entropy ($\bar{S}$) of the polymer segments, which will be related to the transition temperatures by

$$T_{c,m} = (\Delta\bar{U} + P\Delta\bar{V})/\Delta\bar{S} \quad (1)$$

For a salt solution in which $\Delta\bar{S}$ remains unaffected, the change in the transition temperature will be given by

$$\delta T_{c,m} = (\delta\Delta\bar{U} + P\delta\Delta\bar{V})/\Delta\bar{S} \quad (2)$$

We shall neglect the temperature dependence of the partial molal quantities over the range $T_{c,m}$ to $T_{c,m} + \delta T_{c,m}$ and assume that $\delta T_{c,m}$ is solely determined by the salt effect. To calculate $\delta\Delta\bar{V}$ we consider the empty volume which exists in the solvent and which is defined by: observed volume—van der Waals' volume of the molecules⁵. Because of its hydrogen-bonded structure, water is exceptional in having a very large empty volume (about 46%), and Bernal and Fowler⁶ pointed out that the main contribution to the volume change associated with the solution of ions arises from the collapse of this structure. If the polymer can utilise the voids in the solvent, we can write

$$-\delta\Delta\bar{V} = V_e(\text{water}) - V_e(\text{soln}) \quad (3)$$

where $V_e(\text{water})$ is the empty volume existing in the volume of water containing 1 mole of transferring segments and $V_e(\text{soln})$

is the corresponding volume in the salt solution. By considering the transfer of a mole of segments from 1 kg water to 1 kg water + m moles of salt at a given temperature an expression for $\delta\Delta\bar{V}$ in terms of salt concentration can easily be obtained since

$$V = V_e(\text{water}) + 55.5 V_{H_2O} \quad (4)$$

$$m\bar{V}_{\text{salt}} + 55.5 \bar{V}_{\text{water}} = m V_{\text{salt}} + V_e(\text{soln}) + 55.5 V_{H_2O} \quad (5)$$

where V is the observed volume of water, V_{H_2O} and V_{salt} are molar van der Waals' volumes, and $\bar{V}_{\text{salt}}$ and $\bar{V}_{\text{water}}$ are partial molal volumes. Subtracting (5) from (4) gives

$$V_e(\text{water}) - V_e(\text{soln}) = m(V_{\text{salt}} - \bar{V}_{\text{salt}}) + (V - 55.5 \bar{V}_{\text{water}}) \quad (6)$$

In dilute solutions $V \approx 55.5 \bar{V}_{\text{water}}$, so that

$$-\delta\Delta\bar{V} = m(V_{\text{salt}} - \bar{V}_{\text{salt}}) \quad (7)$$

Any change in $\Delta\bar{U}$ brought about by the presence of ions is likely to be due to weak binding of ions to sites on the dissolved segments, and if a Langmuir-type isotherm⁷ is followed we can expect $\delta\Delta\bar{U}$ to be a linear function of m , so that finally we obtain

$$\frac{\delta T_{c,m}}{m} = \frac{(\delta\Delta\bar{U} - P(V_{\text{salt}} - \bar{V}_{\text{salt}}))}{\Delta\bar{S}} \quad (8)$$

Figure 1 shows a plot of $\delta T_c/m$ against $V_{\text{salt}} - \bar{V}_{\text{salt}}$ for the effect of sodium, potassium and ammonium halides on the PVME T_c . With the exception of NH_4Br , these salts have approximately constant values of $\delta T_c/m$ up to 1 M concentration. In the case of NH_4Br an initial slope was estimated from the curved T_c against salt concentration graph in ref. 2. V_{salt} values were calculated from Pauling ionic radii⁸ and

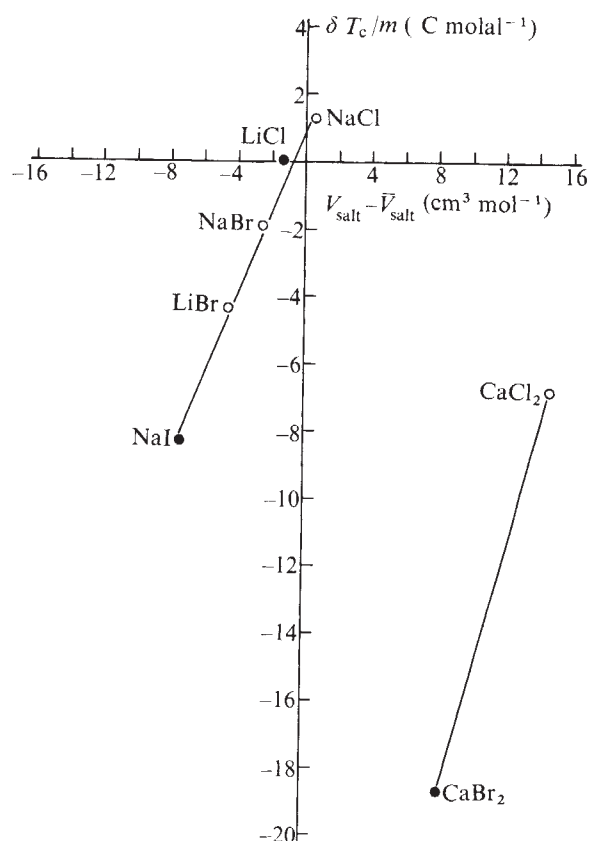


Fig. 2 Salt effects on the thermal denaturation of ribonuclease in aqueous solution. $\circ$, Ref. 3; $\bullet$, ref. 4.

$\bar{V}_{\text{salt}}$ values at infinite dilution and 25 °C were taken from a compilation by Millero⁹. The resulting points fall on three parallel lines, one line for each series of anions with a common cation. This justifies the assumption of constant $\Delta\bar{S}$ and implies that $\delta\Delta\bar{V}$ is dictated by both the cation and anion of a particular salt but that $\delta\Delta\bar{U}$ only depends on the cation. The different intercepts on the $\delta T_c/m$ axis may be interpreted in terms of a greater tendency to ion binding in the order $\text{NH}_4^+ < \text{K}^+ < \text{Na}^+$.

Figure 2 shows a similar plot for the effect of seven salts on the ribonuclease transition. A similar picture is obtained except that the lines have positive slopes because the aggregation process is favoured at low temperatures. Lithium and sodium salts give almost the same positive intercept on the $\delta T_m/m$ axis, whereas the calcium salts have a large negative intercept. Bearing in mind that $\Delta\bar{S}$ is now negative, this can be understood on the basis of a relative binding strength for sites on the protein in the order $\text{Na}^+, \text{Li}^+ < \text{H}_2\text{O} < \text{Ca}^{2+}$. If this interpretation is correct and if this type of plot proves to be general for other ions and macromolecules, the above approach could provide a useful method for separating solvent effects from ion-binding effects. If the interpretation is incorrect, then the interesting empirical observation of common cation lines requires explanation.

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Received May 13; accepted July 7, 1976.

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Structural order in chromatophore membranes of *Rhodospirillum rubrum*

STRUCTURAL studies of the purple membranes of *Halobacterium halobium*^{1–4} have raised the question of whether the in-plane structural order is an exceptional feature of very limited biomembranes or whether it is somewhat more common. We have found that the structural order exists in the chromatophores of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* and the outer membranes of *Salmonella typhimurium*, although extension of the order is limited compared with purple membranes. The present paper reports our results concerning the chromatophores of *R. rubrum*.

Chromatophores were prepared from the carotenoid-deficient blue-green mutant (G-9) of *R. rubrum* by grinding cells with aluminium oxide⁵, a mild treatment used to isolate chromatophores. Oda and Horio⁶ have reported that the chromatophores are vesicles with a diameter of 400–800 Å. Our electron microscopic observations have supported their results. According to Kakuno *et al.*⁷, the chromatophore membrane consists mainly of proteins and lipids, the weight ratios of protein, phospholipid, bacteriochlorophyll and ubiquinones being 1.00:0.24:0.04:0.03. Major protein

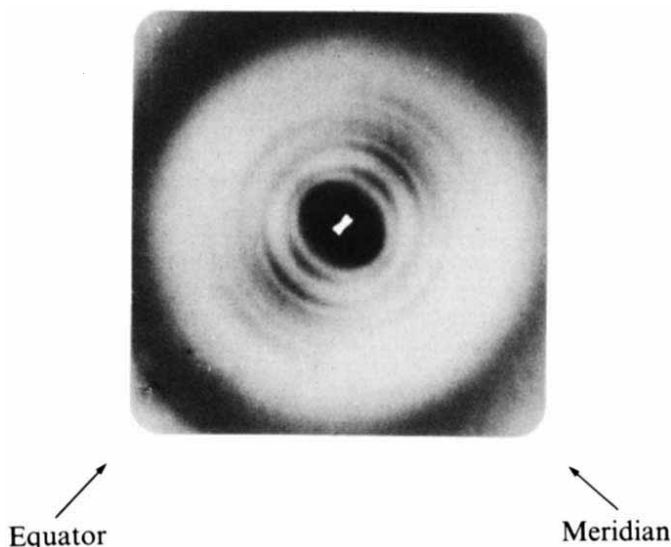


Fig. 1 X-ray diffraction pattern for a dried specimen of chromatophores obtained by grinding cells with aluminium oxide. Specimen-to-film distance 69.3 mm. Exposure time 44 h. See text for equator and meridian.

components are those which have been termed structural proteins by Kakuno *et al.*⁷, and have the molecular weights of 13,000 and 27,000, respectively. The population of the former is about twice the latter (T. Kakuno and T. Horio, personal communication).

Pellets of chromatophores were prepared by centrifugation (for example, at 100,000g for 1 h). Two kinds of specimens were used for X-ray studies: wet pellets which were a dispersion of chromatophores in the solution of 0.1 M Tris-HCl buffer (pH 8.0); and dried specimens which were prepared by placing the wet pellets on a freshly cleaved mica surface, maintained at about 5 °C in a refrigerator for more than 2 d. Strips were then cut off from the dried matter. Each specimen was sealed in a thin-walled glass capillary and its temperature maintained at 4 °C for X-ray exposure. A Rigaku rotating-anode microfocus generator (RU-3HM) was used with a copper target, of which the effective focal spot was $100 \times 100 \mu\text{m}^2$. The camera used was of the Elliott point-focusing type⁸ with a set of double-sector apertures. X-ray diffraction patterns were recorded on Fuji medical X-ray film KX.

All the wet specimens gave diffraction patterns consisting of a series of distinct diffraction rings in the region of the spacing d of $6 \text{ Å} < d < 22 \text{ Å}$ and a sharp ring at $d = 4.16 \text{ Å}$, indicating crystalline packing of hydrocarbon chains in the membranes. All the dried specimens gave diffraction patterns as shown in Fig. 1, when the incident beam was parallel to the plane attached to the mica plate. We have termed the direction parallel to this plane equator and the

Table 1 Observed and calculated spacings d for equatorial reflections

h, k	d calc (Å)	d obs (Å)
0, 1	36.9	—
1, 1	21.3	—
0, 2	18.4	17.9
1, 2	13.9	14.1
0, 3	12.3	12.4
2, 2	10.6	10.5
1, 3	10.2	—
0, 4	9.2	9.4
2, 3	8.5	8.4
1, 4	8.1	—
0, 5	7.4	7.4

Period of 42.6 Å was used for calculations.
 h, k , Miller indices.

perpendicular direction meridian. In Fig. 1 the 4.16 Å reflection (clearly seen on the original film) came out as an equatorial reflection, proving that the membranes lie approximately parallel to the mica plate. The inner equatorial reflections shown in Fig. 1 have a one-to-one correspondence with the inner reflection rings on the photographs of the wet specimens, indicating the existence of some in-plane structural order. There were no appreciable differences between their spacings in the dried and wet specimens. In measuring the spacings, calibration was made using the sodium myristate powder pattern.

The equatorial reflections could be explained by assuming a two-dimensional hexagonal unit cell $a=42.6\text{Å}$. Calculated spacings of reflections are compared with the observations in Table 1.

Wet specimens of chromatophores from which ubiquinones were removed using the isooctane treatment⁹ gave diffraction patterns similar to those of the native chromatophores, indicating that the structural order is related to a regular arrangement of protein molecules. The observed reflections listed in Table 1 had broader widths than those of the purple membranes of *H. halobium* measured with the same camera, implying that the structural order extends only in a limited area of the chromatophore. On the assumption that the protein molecules form small two-dimensional crystalline clusters, the observed line width gave the diameter of the cluster as being about 120 Å, roughly three times the lattice constant a , on the basis of Scherrer's formula.

Dried specimens gave several meridional reflections as seen in Fig. 1. Six meridional reflections were observed for the native chromatophores and eight for the ubiquinone-deficient chromatophores. In the latter case the eight were indexed approximately by assuming a lamellar stacking of membranes with a period of 155 Å, with reasonable accuracy up to the fourth-order reflection; such an indexing was not successful for the native chromatophores. In addition to these, there was an off-meridional reflection with a spacing of 37 Å in both cases. The lamellar reflections and the period of 155 Å suggest that the chromatophores are flattened in the dried specimens, since a thickness for the membrane of $155\text{Å}/2=76\text{Å}$ is plausible.

Similar diffraction patterns indicating structural orders have been found in the chromatophores of *Rps. spheroides* and the outer membrane of *S. typhimurium*. A dried specimen of the former also exhibited lamellar reflections as in the case of *R. rubrum*. Further investigations are in progress.

We thank Professor T. Horio for discussions; and Mr G. Soe and Miss K. Sakata for preparation of chromatophores.

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Bacterial utilisation of organic matter in the deep sea

DISSOLVED organic carbon (DOC) in the deep sea is, after sedimentary humus, the largest reservoir of organic carbon in the hydrosphere. It is utilised primarily by heterotrophic bacteria, which over a long period, in steady-state conditions consume dissolved O_2 from the seawater and produce an equivalent amount of CO_2 by respiration. Knowledge of the rates of O_2 consumption (Table 1) and CO_2 production is useful for quantifying the mixing and circulation of deep-sea water through variations in its O_2 or natural radiocarbon content^{1,2}. The aim of the work reported here was to determine the growth rate of a heterotrophic, low nutrient bacterium isolated from seawater collected at a depth of 2,000 m in the north-central Pacific Ocean (25°03'N, 154°53'W), and to equate this growth rate with O_2 consumption and utilisation of DOC.

We have determined the doubling time in batch culture for this low nutrient bacterium (referred to as 169 and able to grow well in unenriched seawater) in deep-sea water at 5, 13 and 20 °C and under 200 atmospheres of hydrostatic pressure (equivalent to a depth of 2,000 m). The doubling time was 260 h when normalised to the *in situ* temperature of 2 °C using the Arrhenius plot. This growth rate results in a maximum rate of O_2 consumption of $1.4\text{ ml m}^{-3}\text{ yr}^{-1}$, and is discussed in terms of the flux of organic and inorganic carbon between various carbon reservoirs in the deep sea.

The equation for determining bacterial respiration in steady-state conditions was

$$R(\text{ml O}_2\text{ m}^{-3}\text{ yr}^{-1}) = \frac{1.6 \times 10^{10} A(100-E)}{D},$$

$$(1\text{ ml O}_2\text{ m}^{-3}\text{ yr}^{-1}) = 0.44\text{ mg C m}^{-3}\text{ yr}^{-1}$$

where R is respiration, A is biomass of synthesised microbial cell carbon (g), E is percentage of carbon assimilated, $100-E$ is percentage of carbon respired, and D is doubling time of microbial cells (h). The factor 1.6×10^{10} takes into account the normalisation of doubling times of hours to years, and conversion of g carbon to ml of O_2 . A was calculated using the following expression

$$A = 10^{-13} NV$$

where N represents numbers of bacteria per l, V is volume of each bacterium (μm^3), and 10^{-13} is the weight of one bacterium of volume $1\text{ }\mu\text{m}^3$ expressed as g of carbon. Each bacterium is assumed to be 80% water and 10% carbon. In our calculations, bacterial volumes were determined from many enumerations of bacteria in deep-sea samples, either by direct microscopy or with the scanning electron microscope (SEM). SEM observations were made on cells fixed *in situ*. We used cell volumes calculated for cocci (0.2 and 0.5 μm diameter) and rods (0.2×0.5 and $0.5 \times 1.0\text{ }\mu\text{m}$). Numbers of bacteria per l in the central Pacific Ocean deep water were obtained from two sources^{4,7}, and from our own observations. These values ranged from 10^6 to 10^7 l^{-1} .

The percentage of carbon assimilated (E) in the above equation is difficult to estimate for deep-sea bacteria *in situ*. Most terrestrial heterotrophic bacteria studied in the laboratory in enriched conditions show 10–20% efficiency. Sorokin⁴ found that 40% and Jannasch⁸ 95% of acetate added to seawater (100 and 2,000 mg l^{-1} , respectively) was assimilated by deep-sea bacterial populations.

To calculate R , we used combinations of the above cell volumes, and assumed that the carbon cycle in the deep sea, including bacteria, is in a steady state (otherwise, there would

Table 1 Utilisation of organic carbon in the deep sea

Method	Depth (km)	Location	DOC and/or POC utilisation (mg C m ⁻³ yr ⁻¹)*
Calculated from a vertical diffusion ¹ advection model	1-4	Central Pacific	About 1.8 (4.1)
Calculated from a vertical diffusion ² advection model	1-4	South Pacific	About 0.67 (1.5)
<i>In situ</i> incubation with ¹⁴ C-labelled ³ substrates	5	North-east Atlantic	2-13 (4.6-29.6)
<i>In situ</i> incubation with ¹⁴ C-labelled ⁴ substrates	4	Western Pacific	11.2-33.6 (25.6-76.7)
O ₂ uptake by electron transport ⁵ system	1-6	Eastern tropical Pacific	About 0.70 (1.6)
Adenosine triphosphate ⁶ content of cells	1-4	North-east Pacific	About 0.44 (1)
This report	2	North-central Pacific	0.04-0.61 (0.09-1.4)

Calculated and experimental values are given. Equivalent O₂ consumption rates are based on 1.0 ml O₂ consumed = 0.44 mg C utilised, assuming a C/N ratio by atoms of 10 in deep-sea DOC and POC.

*In parentheses: O₂ consumption (ml O₂ m⁻³ yr⁻¹).

be a net increase in biomass); we therefore assumed 100% of DOC to be respired; that is $E = 0$. The range of values for R obtained from these combinations was 0.09-1.4 ml O₂ m⁻³ yr⁻¹ (0.04-0.61 mg C m⁻³ yr⁻¹). But from what seems to be the most reasonable assumptions for N and V (3.8×10^6 l⁻¹ and 0.26 μm³ respectively), a value of 1.4 ml O₂ m⁻³ yr⁻¹ (0.61 mg C m⁻³ yr⁻¹) for R is the most realistic and represents the upper limit of R . In this calculation we treated all bacteria in the deep sea as growing in a manner similar to that of low nutrient bacteria. This is probably not true; some bacteria may be less, and some more active, than bacterium 169.

Previous *in situ* measurements of bacterial uptake of DOC^{3,4} were based on high concentrations of added substrate and so may not be relevant to measurements of the actual activities of deep-sea bacteria. Because little is known of the chemical composition of the DOC in deep water, the estimates for utilisation of DOC we have obtained using the natural substrate are more appropriate for calculating organic carbon fluxes and rates of utilisation of O₂ in the deep sea than rates derived from experimental work with added substrates. The particular fraction of the DOC which is utilised by low nutrient bacteria may be organic material of a more labile nature than the 'old', refractory DOC constituting the bulk of the organic matter, but we cannot judge which fraction is utilised.

This determination of organic carbon utilisation, 0.61 mg C m⁻³ yr⁻¹ (1.4 ml O₂ m⁻³ yr⁻¹ consumed), may be used in a qualitative way to estimate the organic carbon balance at 2,000 m in the north-central Pacific. If we assume a steady state, ($-dDOC/dt = -dPOC/dt = -dO_2/dt = +dCO_2/dt = 0$), and concentrations of DOC (unpublished results from cruise Cato-1, Institute of Marine Resources, University of California, San Diego, June-July, 1972), POC, particulate organic carbon⁸ and O₂ (ref. 9) of 450 mg m⁻³, 3 mg m⁻³ and 1.7 l m⁻³, respectively, then the residence time of DOC, POC and O₂ would be 738, 4.9, and 1,214 yr, respectively. This calculated DOC residence time of 738 yr is three to five times lower than the value of 2,600-3,500 yr determined by natural radiocarbon measurements of the DOC at 2,000 m in the north-east Pacific^{10,11}. This suggests that R is less than 1.4 ml O₂ m⁻³ yr⁻¹ and that the rate of utilisation of DOC is > 0.1 and < 0.5 mg C m⁻³ yr⁻¹. The POC would be utilised in ~ 5 yr if the entire microbial population is using POC and not DOC as a substrate. This is unlikely for two reasons: first, the percentage of attached, non-filterable bacteria compared with the free-living population is 25% or less⁷, and second, the consumption of POC by bacteria during the estimated mean time of 2 yr it takes the POC to sink from 500 to 2,000 m (ref. 11) would be 1.22 mg C m⁻³ (2R). Such a rate of utilisation would require a gradient (decreasing concentration) of POC from 500 to 2,000 m. This is not observed in the north-central Pacific from 500 m to the

bottom, where the concentration of POC is essentially invariant⁸. It thus seems that DOC is the main source of energy for the low nutrient, heterotrophic bacteria in the deep water of the north-central Pacific, and that the rate of utilisation of organic carbon or consumption of O₂ we have measured is of the same magnitude, and probably less, than earlier experimental estimates of a more indirect nature given in Table 1. Indeed, if the rates of O₂ consumption were much greater than 0.6 ml O₂ m⁻³ yr⁻¹, vast areas of the deep sea would be anoxic.

Finally, the results reported here support the validity of using vertical-diffusion, vertical-advection models^{1,2} for calculating such non-conservative properties such as O₂ consumption and carbon utilisation in the deep sea.

We thank Ms S. L. Shimp for technical assistance. This work was supported by the ERDA.

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Received April 27; accepted July 14, 1976.

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Circadian rhythm of anaerobiosis in a polychaete annelid

ALTHOUGH intertidal organisms which cannot breathe air must be regularly subjected to tidal conditions in which they must respire anaerobically, there are no known cases in which these or other animals reduce environmental conditions to the point at which they must respire anaerobically. Indeed such reduction of microhabitat conditions would not be expected because of the relative inefficiency of known anaerobic energy-generating pathways. This report describes a rhythm of alternating aerobic and anaerobic metabolism

in the polychaete *Nereis virens*. The rhythm is circadian under low intensity constant illumination.

Burrows of *N. virens* are ventilated with undulatory movements of the body which serve to draw well-oxygenated water and food into the burrow. I have conducted long term monitoring of the ventilation rhythms of animals adapted to 10.0-ml synthetic burrows¹. Individuals have been maintained in synthetic burrows in the laboratory for periods of up to 20 months. Ventilation activity was measured with a directly heated thermistor flowmeter inserted through the wall of the synthetic worm burrow and separated from the animal by a nylon screen¹.

Twelve individuals were tested in continual light (LL 12:12), eight individuals were tested in a light-dark regime of 12 h light and 12 h dark (LD 12:12), and six individuals were tested in continual darkness (DD 12:12). A total of 325 d of continuous recording was obtained for the 26 animals tested. Both incandescent and fluorescent light sources were used, and light intensity was maintained at approximately 3,000 lx at the burrow. Before experimentation, all animals (5.0–6.0 g) were maintained on a daily cycle of 14 h light and 10 h darkness at $10 \pm 2^\circ\text{C}$ for 5–70 d. At the start of an experiment individuals in burrows were transferred to a 15-l tank (10°C) which received a continuous flow of aerated seawater. The thermistor was inserted through the wall of the worm burrow near either end. Vibration and noise from activity in the laboratory were minimised. Several animals were tested in closed as well as open-system conditions to prevent possible influence of chemical time-cues from entering the seawater system.

Ventilation for each hour of each day was measured in relative volume units by determining the areas under the curves of the continuous activity records. Period lengths of ventilation rhythms were estimated using the periodogram procedure of Enright² which is particularly appropriate for

serially correlated time series data. The experimental amplitudes for a series of computer-generated assumed period lengths are compared; the measure of amplitude is the standard deviation of the hourly means of activity (area under the activity records). The plot of amplitude estimates against the assumed period length is a periodogram, and the period value with a large amplitude represents the most likely correct period length for the observed function. All ventilation rhythms were analysed for likely period lengths from 3 to 33 h in 0.1-h increments.

Burrow $p\text{O}_2$ was monitored continuously with a 1.0-mm polarograph (IBC) during long term ventilation recordings. Burrow pH was monitored continuously with a combination microelectrode (Bolab) inserted through the wall. Levels of lactate in burrow water and tissue were determined enzymatically according to the appearance of NADH at 340 nm (ref. 3). In separate experiments groups of six worms were placed in 10-ml sealed burrows and sampled for either tissue lactate or burrow water lactate after a chosen period of forced burrow closure. Respiratory rates were determined separately for 22 individuals placed in air-saturated seawater (10°C), after various periods of forced closure in 10-ml synthetic burrows.

While I have observed short term (3–20 min) cycling of ventilation and rest periods similar to those observed for other polychaetes^{4–9}, I have also observed longer rest periods (>1 h) superimposed on the short term irregular pattern. These long rest periods (up to 9 h) create anaerobic burrow conditions and appear in recordings only after 2–4 d. In conditions of constant illumination, the ventilation activity shows a circadian periodicity (Fig. 1a and b). While one of 12 animals tested in LL was aperiodic, the average observed period length was $21.3 \pm \text{s.d. } 0.65 \text{ h}$ and the average duration of rest periods >1 h was $3.85 \pm 2.4 \text{ h}$. While all animals tested in LD and DD were aperiodic (Fig. 1c–f),

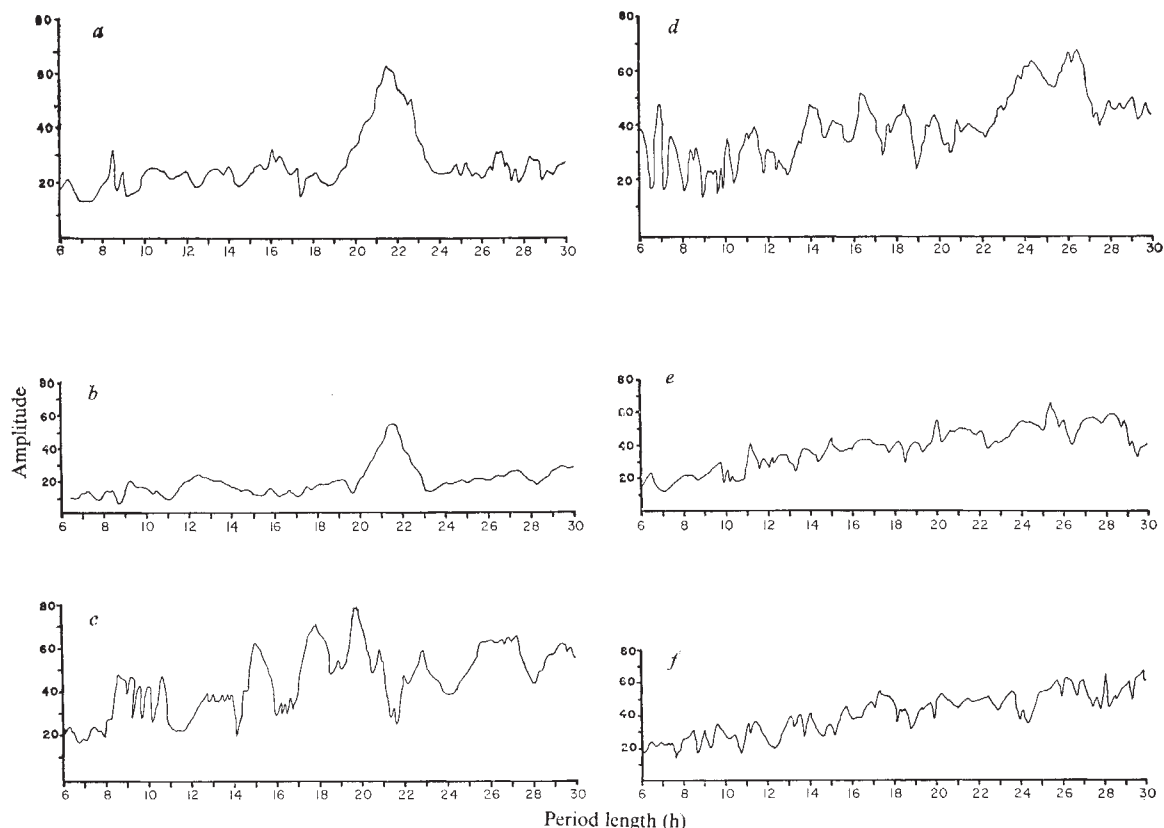


Fig. 1 Typical periodograms of burrow ventilation. a, b, Periodic anaerobiosis under constant illumination; c, d, aperiodic activity under light-dark cycle; e, f, aperiodic activity in constant darkness.

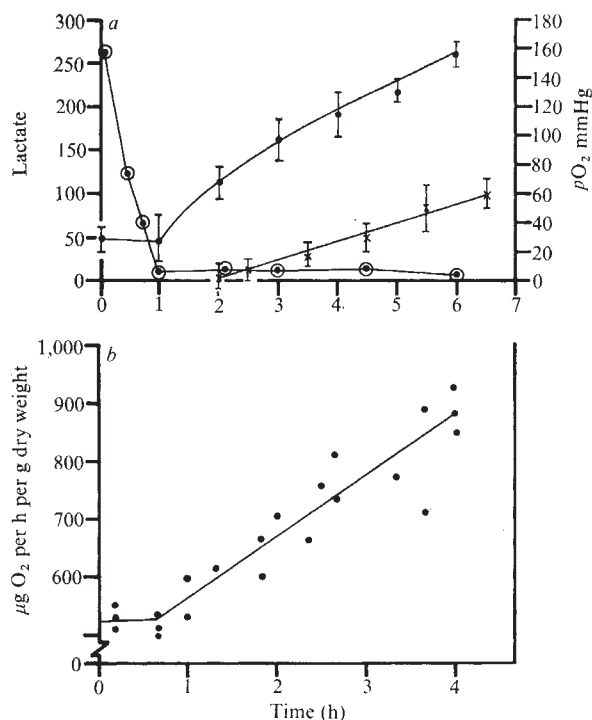


Fig. 2 *a*, Tissue lactate (●, μg per g wet tissue $\pm$ s.e., $n = 6$), burrow lactate (x, μg per ml burrow water $\pm$ s.e., $n = 6$), and pO_2 (circle and dot) as a function of length of rest period ($n = 8$). *b*, Maximum oxygen consumption following various lengths of rest periods ($n = 22$). Tissue lactate, burrow lactate and respiratory debt were determined for groups of six animals placed in sealed 10-ml burrows ($10^\circ C$) for predetermined times, since it was impractical to obtain tissue or water samples during the rest periods observed in the ventilation studies. The pH records obtained from burrows during spontaneous rest periods of the ventilation studies agree closely with those obtained at the end of the forced-closure experiments.

long rest periods were nevertheless observed in both of the latter conditions. The average lengths of rest periods >1 h in the LD and DD groups were 3.39 ± 1.45 and 3.13 ± 2.0 h, respectively. The frequencies of occurrence of rest periods >1 h varied for animals tested in LL, LD and DD and were 1.15 ± 0.21 , 0.60 ± 0.14 and 0.52 ± 0.10 per d, respectively. Furthermore, the DD group spent an average of 71% of each day involved in ventilation activity while the LL and LD groups averaged 21 and 35%, respectively.

Measurements of oxygen consumption, lactate and pH within the polychaete burrow indicate that during rest periods, the worms respire anaerobically, accumulate small amounts of lactic acid and later pay an oxygen debt.

At a burrow oxygen tension of 5–10 mmHg ($10^\circ C$), detectable aerobic respiration ceased and presumably there was a switch to anaerobic pathways. Lactate appeared in the sealed burrow after a 2-h rest period and reached $92 \mu g ml^{-1}$ after 6.5 h (Fig. 2*a*). Increased tissue lactate levels were evident after about 1 h, which is approximately the time required to reduce the burrow pO_2 to 5–10 mmHg, and reached approximately $260 \mu g$ per g wet tissue after 6 h. When a rest period ended and the burrow was again ventilated, the oxygen debt was repaid in part, as indicated by increased respiratory rates (Fig. 2*b*), which were directly related to the length of the period of forced closure.

Continuous monitoring of burrow pH during ventilation studies showed that burrow acidity was a function of the length of the rest period, with pH on one occasion reaching a low value of 5.97 after 8.2 h of rest. Organic acids other than carbonic and lactic must account for such observed depressions in pH because, in theory, pH could be reduced only from 8.1 to about 7.0 by animal respiration.

In summary, *N. virens* seems to cease burrow ventilation

for periods of up to 9 h during which respiration continues anaerobically and the worms accumulate lactic and other organic acids. It is doubtful that the vascular haemoglobin serves an oxygen storage function during extended tidal exposure⁵.

Newell¹⁰ has suggested that temperature-independent resting metabolism (O_2 consumption) is associated more often with animals found intertidally than with those found subtidally, although two polychaete species have resting metabolic rates that are temperature dependent¹¹. The observation of a spontaneous creation of anaerobic burrow conditions is important since normal rest metabolism of many invertebrates may include a significant non-oxidative component involving accumulation of lactate or other partially oxidised products of the Embden–Meyerhoff pathway. Any function of the observed periods of anaerobiosis, as well as the role of low intensity illumination in synchronising the anaerobic periods is as yet unknown.

It is interesting that the bivalve *Arctica islandica* has been shown to burrow aperiodically into sediments and respire anaerobically¹².

I thank A. H. Price, II and S. Kraul for review of the manuscript. This work was supported in part by NOAA Office of Sea Grant.

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Received May 4; accepted July 6, 1976.

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Plutonium isotope ratios in polychaete worms

We have noted with interest recent reports which suggest a preferential biological availability of ^{238}Pu over $^{239+240}Pu$ in terrestrial and aquatic organisms^{1–4}. Although kinetic isotope effects do occur in biological systems for low mass number elements (H, C, N), such effects are generally discounted as the mass number of the element increases. Thus, differential biological availability of isotopes of high mass number elements, such as plutonium, would normally be attributed to differences in the chemical or physical forms of the isotopes or to the different weights of isotope available to organisms as a result of large differences in their specific activities. These arguments have indeed been used to explain differential plutonium isotope behaviour in animals in controlled laboratory conditions^{5–7}. It is not certain, however, that the same arguments can be used to explain anomalies of plutonium isotope behaviour in organisms contaminated by nuclear test debris or by wastes from nuclear fuel reprocessing plants. Plutonium activity levels, while easily measurable, do not approach those generally used in laboratory studies. In addition, geochemical weathering could conceivably alter any original differences in physical or chemical form of the plutonium. We report here the results of experiments which show that deposit-feeding marine worms living in sediments contaminated with plutonium isotopes in different ways do not preferentially accumulate ^{238}Pu over $^{239+240}Pu$. This is particularly significant in that the worms held in sediments contaminated in the natural environment may have been exposed to different chemical and physical forms of plutonium isotopes.

Table 1 Plutonium isotope ratios in polychaete worms and sediment

Sediment sample	Mean $^{238}\text{Pu}/^{239+240}\text{Pu}$ ratios	
	Worms	Sediment
Mediterranean	0.0076 ± 0.0030 (4)	0.0082 ± 0.0006 (5)
Bravo Crater	0.10 ± 0.02 (4)	0.082 ± 0.004 (5)
Windscale	0.25 ± 0.04 (3)	0.231 ± 0.004 (4)

Errors associated with the ratios are propagated at the 2σ confidence interval. Numbers in parentheses indicate number of samples used in calculating mean ratio.

We exposed polychaete worms (*Nereis diversicolor*) to laboratory-labelled sediment, sediment collected from a former weapons test site and sediment contaminated by wastes from a nuclear fuel reprocessing plant, in an attempt to observe any preferential uptake of ^{238}Pu over $^{239+240}\text{Pu}$. To prepare our laboratory-labelled sediment, we added 91 nCi of $^{239+240}\text{Pu}$ (containing trace amounts of ^{238}Pu) in either the +4 or +6 oxidation state to 1 l of Millipore-filtered ($0.45 \mu\text{m}$) Mediterranean seawater with constant stirring. Some 200 ml of Mediterranean sediment was then introduced into the seawater and the slurry was allowed to mix for 2 d. After settling, the seawater was decanted and several fresh seawater washes were performed to remove any plutonium not bound to the sediments. A portion of a core sample (23–49 cm deep) collected from Bravo Crater at Bikini atoll and several hundred grams of surface sediment collected from the Irish Sea near the Windscale fuels reprocessing plant on the Cumbrian coast were equilibrated for several days with filtered Mediterranean seawater in a manner identical to that of our laboratory-labelled sediment. Aliquots of all sediment samples had easily measurable quantities of ^{238}Pu and $^{239+240}\text{Pu}$ ($\sim 2 \times 10^4 \times$ higher than fallout levels in coastal ocean sediments for the lowest activity sample) and homogeneity was good. Since the Bravo Crater sediment was contaminated as the result of testing a large thermonuclear device (1954) we tested the homogeneity of plutonium isotopes further by leaching an aliquot of the sample with a dilute solution of EDTA at pH 3 and determined the $^{238}\text{Pu}/^{239+240}\text{Pu}$ ratio in portions of the leachate with time. No differences in isotope ratios were observed, suggesting that both small and large particles of the sediment were homogeneous with respect to both isotopes.

Worms introduced into the sediments were allowed to accumulate plutonium for between 5 and 40 d. At prescribed times during this interval, 5 to 31 individuals were removed from the sediments, rinsed several times in clean seawater and placed in uncontaminated Mediterranean sediments for 24 h, where they were allowed to void their gut contents by ingesting sediment. Finally, all sediment was removed from the organisms by placing them on a mat of wet algae for a further 24 h. Radiochemical analyses of both worms and sediment were accomplished by standard techniques⁹ designed to ensure the radiochemical purity of the separated plutonium. Isotope ratios were determined by α -spectrometric techniques.

Table 1 sets out the results of the isotope ratios observed in the worms and the sediments to which they were exposed. Since no significant differences in isotope ratios were observed in worms killed at different times (5, 9, 15, 25, 40 d), we have averaged all results for each sediment type. The somewhat unsatisfyingly large error terms associated with the Mediterranean and Bikini worm samples result from the extremely low levels of plutonium ultimately taken up by these organisms⁹ and the low initial $^{238}\text{Pu}/^{239+240}\text{Pu}$ ratio in the sediments. Counting times for many samples approached 10 d.

Our results indicate that at least for the polychaete worms studied, no preferential uptake of ^{238}Pu over $^{239+240}\text{Pu}$ occurs whether the sediment is labelled with known chemical forms of plutonium or whether the sediments contain plutonium from aged nuclear test debris or plutonium from fuel reprocessing wastes. This is in contrast to the near twofold magnification of ^{238}Pu over $^{239+240}\text{Pu}$ observed between plants and organisms with respect to sediment in a freshwater ecosystem contaminated

by nuclear fuel processing wastes⁴ and the near 20-fold magnification observed between rodent tissue and soil in a terrestrial landscape contaminated by fallout². Although the reasons for these disparities await clarification, general statements regarding preferential bioavailability of ^{238}Pu over $^{239+240}\text{Pu}$ after its introduction into different ecosystems should be viewed cautiously. It is moreover worth emphasising that naturally occurring α -emitting radionuclides can follow plutonium in any radiochemical separation scheme, and unless scrupulous attention is paid to the radiochemical purity of the plutonium isolation technique, erroneous values for ^{238}Pu can result⁸. Clearly, such considerations become important for environmental samples where plutonium activity levels are low.

We thank N. Mitchell and F. Lowman for samples and V. T. Bowen for suggesting the EDTA leach experiment. The International Laboratory of Marine Radioactivity operates under a tripartite agreement between the International Atomic Energy Agency, the Government of the Principality of Monaco and the Oceanographic Institute at Monaco. Support for this work is gratefully acknowledged.

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Received May 4; accepted July 7, 1976.

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Occurrence of plutonium and americium in plaice from the north-eastern Irish Sea

SMALL amounts of transuranic nuclides are discharged routinely into the north-eastern Irish Sea from the nuclear fuel element reprocessing plant at Windscale, Cumbria. Regular monitoring of the marine environment, as part of the established control procedure is made by the Ministry of Agriculture, Fisheries and Food^{1,2}, primarily to ensure the radiological safety of the discharges in public health terms. Data on the partitioning of plutonium and americium between seawater, suspended material and sediment have already been published^{2,3} and the purpose of this communication is to present a preliminary report of these radionuclides in the plaice (*Pleuronectes platessa*). This is a demersal fish of commercial importance in the area and the critical material for discharges of transuranic nuclides. There are few published data on the concentrations of plutonium in marine fish⁴ and no data for americium have been reported.

During 1975 four catches of plaice were made approximately 5 km south of the pipeline. Thirty fish—principally group III, 25–30 cm long—were selected from each catch and their dissected organs were bulked and ashed. Analysis for the plutonium isotopes was based on the method of Wong⁵ except that concentration was obtained initially by coprecipitation on calcium oxalate and subsequently on neodymium hydroxide. Separation of americium from plutonium, and purification of the latter, was effected by anion exchange from a hydrochloric acid medium. The americium was separated from lanthanides by anion exchange at 80 °C using ammonium thiocyanate. Both

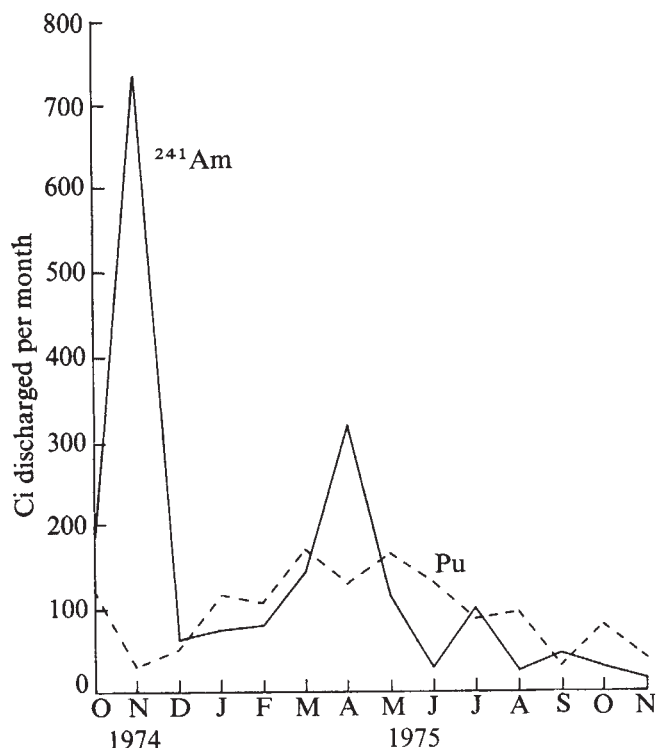


Fig. 1 Monthly discharges of ^{238}Pu , ^{239}Pu , ^{240}Pu and ^{241}Am from Windscale for the period October 1974–November 1975 (from returns made by British Nuclear Fuels Ltd).

plutonium and americium were electrodeposited from a slightly acid ammonium sulphate medium on to stainless steel disks. Radionuclide content was measured by α spectrometry using a 128-channel pulse height analyser and a silicon surface barrier detector (100 mm²) to ensure adequate separation of the isotopes from the yield tracers ^{238}Pu and ^{243}Am . Chemical recoveries were > 70%. The results, $\pm 2\sigma$ counting error, are given in Tables 1 and 2.

The highest concentration of plutonium in the internal organs of the fish collected in February was in the kidneys. Concentrations in the kidneys of fish caught later in the year, however, were exceeded by those in the gut. Plaice feed little during the winter and most of those caught in February were void of gut contents. The other three samples all contained various food species, consisting largely of *Amphiura filiformis*, which was particularly dominant in the sample collected in May, and several lamellibranchs including *Ensis ensis*, *Abra alba*, *Cultellus pellucidus* and *Nucula turgida*. Less frequent were *Nephtys*, *Pectinaria* and *Aphrodite*. The predominance of these species suggests that the fish had been feeding in an area of muddy sand, and not inshore of the pipeline⁶. It is interesting that although during the summer the food intake was greatly increased, and the discharges of plutonium were somewhat

Table 2 ^{241}Am in organs of the plaice

Organ	Date of sample collection			
	February 18, 1975	May 13, 1975	August 12, 1975	November 11, 1975
Kidney	440 $\pm$ 50	103 $\pm$ 15	65 $\pm$ 5	120 $\pm$ 14
Liver	590 $\pm$ 120	186 $\pm$ 29	35 $\pm$ 6	54 $\pm$ 6
Gut	160 $\pm$ 20	89 $\pm$ 14	114 $\pm$ 22	122 $\pm$ 17
Gut contents	930 $\pm$ 600	6,600 $\pm$ 540	4,940 $\pm$ 270	7,390 $\pm$ 36
Gill	210 $\pm$ 60	128 $\pm$ 23	310 $\pm$ 40	89 $\pm$ 7
Skin	20 $\pm$ 8	15 $\pm$ 2	6.2 $\pm$ 2.8	11 $\pm$ 0.9
Bone	110 $\pm$ 50	33 $\pm$ 9	28 $\pm$ 6	2.1 $\pm$ 0.5
Muscle	2.3 $\pm$ 0.5	1.4 $\pm$ 0.2	0.9 $\pm$ 0.2	0.3 $\pm$ 0.08

Results are expressed in fCi per g wet weight.

higher during the early summer (Fig. 1), neither the concentration of plutonium in the liver, nor that in the muscle, increased significantly. The concentrations in the gut wall, although higher in the samples taken after February, were about two orders of magnitude lower than the gut contents. Concentration by the gill was highest in the samples collected in May and August, although external contamination may have been variable in spite of their being well rinsed in clean water during dissection.

The concentrations of ^{241}Am in the internal organs were all generally higher than those of plutonium, the ratios being somewhat higher than those of the gut contents for May, August and November. The ^{241}Am data are also interesting in that the concentrations in kidney, liver, muscle and bone decreased markedly from February to August and may well have been influenced by the relatively large release during November 1974 (Fig. 1). It is not certain, however, that the fish sampled during the year have been taken from the same population.

Sufficient material was obtained from the fish in November to analyse gonad tissue. The bulked testis sample contained 0.64 ± 0.3 fCi per g wet weight $^{239+240}\text{Pu}$ and < 0.5 fCi per g wet weight ^{241}Am . In contrast, the ovaries contained 1.5 ± 0.5 fCi per g wet weight $^{239+240}\text{Pu}$ and 9.3 ± 3.2 fCi per g wet weight ^{241}Am .

In view of the fluctuating discharges (Fig. 1) it is evident that a steady-state body burden will not exist for fish caught close to the pipeline. An accurate estimation of a concentration factor over seawater therefore cannot be made, and would in any case depend on the location of the seawater sample and the movements of the local population of plaice. Seawater samples were taken from the area fished during July 1975 and contained ≈ 1 pCi l⁻¹ of $^{239+240}\text{Pu}$ and ≈ 0.2 pCi l⁻¹ of ^{241}Am in the filtrate (< 0.22 μm) (personal communication from D. F. Jefferies). Thus the approximate concentration factors over seawater (filtrate) for the samples of muscle collected in August are of the order of < 1 for $^{238+239}\text{Pu}$ and ≈ 5 for ^{241}Am . The data obtained also emphasise the very low levels of plutonium and americium in the edible fraction of Irish Sea plaice taken from the area most liable to contamination; the concentrations measured in fish muscle are equivalent to less than 0.01% of

Table 1 Plutonium in organs of the plaice

Organ	Date of sample collection							
	February 18, 1975		May 13, 1975		August 12, 1975		November 11, 1975	
	$^{239+240}\text{Pu}$	^{238}Pu	$^{239+240}\text{Pu}$	^{238}Pu	$^{239+240}\text{Pu}$	^{238}Pu	$^{239+240}\text{Pu}$	^{238}Pu
Kidney	88 $\pm$ 32	7.0	18 $\pm$ 6	4.9	20 $\pm$ 4	3.2	44 $\pm$ 5	3.9
Liver	29 $\pm$ 9	3.9	9.1 $\pm$ 2.8	7.5	5.3 $\pm$ 0.9	4.5	18 $\pm$ 3	3.9
Gut	15 $\pm$ 5	3.9	35 $\pm$ 7	5.4	72 $\pm$ 4	4.4	66 $\pm$ 4	4.4
Gut contents	540 $\pm$ 30	4.8	3,570 $\pm$ 250	4.0	2,710 $\pm$ 180	4.5	5,010 $\pm$ 200	4.3
Gill	13 $\pm$ 4	—	450 $\pm$ 39	4.1	770 $\pm$ 50	3.8	38 $\pm$ 8	1.6
Skin	3.2 $\pm$ 1.5	4.2	4.2 $\pm$ 1.5	4.1	4.3 $\pm$ 0.6	4.0	2.9 $\pm$ 0.4	3.4
Bone	14 $\pm$ 5	3.6	2.9 $\pm$ 1.1	6.1	2.8 $\pm$ 0.9	1.5	0.65 $\pm$ 0.07	4.6
Muscle	0.16 $\pm$ 0.04	—	0.34 $\pm$ 0.05	3.1	0.47 $\pm$ 0.07	4.8	0.17 $\pm$ 0.03	2.3

Results are expressed in fCi per g wet weight.

the derived working limit, as defined by the International Commission on Radiological Protection^{7,8}, based on calculations using a dose limit to the public of 3 rem per yr and a maximum rate of consumption of 300 g of fish per d.

Part of the work was supported by contract with the European Atomic Energy Community.

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Received May 7; accepted July 6, 1976.

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Presence of a buried mammalian carcass indicated by fungal fruiting bodies

Hebeloma vinosophyllum Hongo is a terrestrial agaric originally found in broad-leaved and conifer forests but not described as having any special ecological requirements¹. It now seems that this species often fruits where a dead mammal has been buried.

On August 24, 1968 I found a group of luxurious fruiting bodies of this species in a stand of *Castanopsis cuspidata* at Iwakura, Kyoto. The fruiting bodies appeared to be well nourished and covered an area of about 1,500 cm². The surface of the ground did not seem to have been disturbed. This fungus had been found to fruit well only when the ground in forests has been dressed with urea or a similar material

which releases ammonia on decomposition². The presence of fruiting bodies in an untreated area suggested that there might have been a source of nitrogen in the soil. At a depth of less than 10 cm I found the remains of a dog carcass, which consisted of bones and decayed skin, and the soil around the carcass contained abundant white mycelia.

On October 8, 1975 a single well developed fruiting body of the same species was found in a stand of *Pinus densiflora* at Yoshida-yama near the Kyoto University campus. As before, the ground appeared to be superficially normal but some pieces of cat bone were found in the top 20 cm of soil. The soil and bones were replaced and on October 30 another fruiting body appeared (Fig. 1a). The bones were removed from the soil and are shown in Fig. 1b. On this occasion denatured fatty tissue was found and there was a smell of decaying animal matter. As before, no flesh remained and the soil was whitish with mycelia. In May to June 1976, several fruiting bodies appeared again on the same spot.

These facts do not exclude the possibility that this species colonises normal (untreated) soils. It is often very difficult, however, to determine whether or not a soil is really normal, since human urine and faeces, which also yield this fungus, leave nothing after decomposition. It is well known that entomogenous fungi or caterpillar fungi occur directly on the bodies of some invertebrates but there are no reports of indirect (soil-mediated) occurrences of agarics on mammals. The present finding may lead to speculation that a victim of homicide buried in soil could be located using the fruiting bodies of this or some other fungi, assuming the burial was shallow. (In forensic medicine, 'filamentous fungi' or 'fungal hyphae' are known to occur sometimes directly on human corpses or in graves³.) After urea or ammonia application this species appears after 3-8 months and continues to fruit over a period of 1-2 yr, forming part of a succession, all the members of which respond to this treatment, and are called ammonia fungi². It is not known whether this species could be used to determine how long a corpse has been buried.

The closely related species *Hebeloma sarcophyllum* Peck (= *H. porphyrosporum* R. Maire) is found in Europe, North Africa and North America⁴. It would be interesting to know if this species has similar ecological requirements.

I thank Dr R. F. O. Kemp, University of Edinburgh, for reading the manuscript.

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Received June 24; accepted July 7, 1976.

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Fig. 1 a, Fruiting body of *H. vinosophyllum* on the superficially normal ground. b, Fruiting body together with cat bones taken from soil under that fruiting body.

Appetitive learning, patterned alternation, and extinction in 10-d-old rats with non-lactating suckling as reward

HUGHLINGS-JACKSON¹ has expressed the view that, during ontogenesis, neural centres responsible for inhibition develop more slowly than centres responsible for excitation. Since then, the working hypothesis has been that the later-developing, phylogenetically 'higher' neural centres act to

regulate (inhibit?) the excitation of the 'lower' centres. There has been some behavioural and physiological support for this position. In altricial mammalian species the hippocampus and neocortex become functionally mature relatively late in development², and the hippocampus, in particular, has been implicated in the expression of behavioural inhibition³⁻⁵. Preweanling rats, during weeks 3 and 4 of life, seem to lack maturity for tasks requiring suppression of responding; for example, they are deficient in passive avoidance⁶ learning and in habituating to novel stimuli^{7,8}. Their performance in excitatory tasks, such as active avoidance⁹ and approach for food¹⁰, seems to be less deficient. These task-specific deficits resemble those shown in hippocampally lesioned adult animals¹¹. Campbell *et al.* have noted the correspondence between later-developing inhibitory centres in the rat and the decline in spontaneous activity occurring during ontogenesis¹², and have demonstrated that psychopharmacological agents¹³ and brain lesions¹⁴ specific to inhibitory systems are ineffective in increasing behavioural arousal early in development. In our

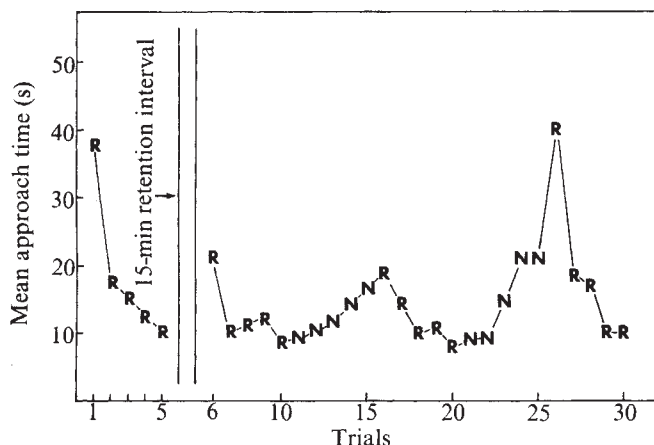


Fig. 1 Mean approach time for animals in experiment 1.

laboratory, experiments on instrumental appetitive learning in preweanling rats have involved the use of dry food pellets as reinforcers in pups as young as 17 to 20 d of age. In animals as young as this, we have demonstrated acquisition, extinction, immediate persistence, and long term persistence using procedures that are similar to those for adult rats¹⁵⁻¹⁷. We now report two experiments demonstrating behavioural plasticity in the 10-d-old rat, involving acquisition and subsequent extinction of an approach response where reward is the opportunity to suck briefly from an anaesthetised mother rat.

Our first experiment established that infant rats will quickly learn to approach when the reward is an opportunity to attach to a nipple of an anaesthetised mother rat for a brief interval, and that this learned tendency to approach can be successively weakened and strengthened by making the mother inaccessible and accessible on alternating blocks of trials. Our second experiment provided data from acquisition and extinction for two groups of subjects which differed in their schedule of reward in acquisition. The general procedure in both experiments, including deprivation treatment, was as follows: On the morning of day 10 pups from litters culled to 8 at birth were separated from their mother 8 h before training by being placed in a Plexiglas chamber partially filled with pine shavings. Temperature in the chamber was maintained at 37 °C (the average temperature in an undisturbed nest) using thermostatically controlled commercial heating pads placed under the Plexiglas floor. Experimental training was conducted in

a clear Plexiglas alley (32 cm long and 8 cm wide) also maintained at 37 °C. Approximately 20 min before the first trial, the mother received intraperitoneally a 2 ml kg⁻¹ injection of EquiThesin, a general anaesthetic producing a surgical level of anaesthesia and blocking milk release.

Preliminary training in both experiments consisted of placing each pup separately against the mother and allowing it to attach to a nipple for 15 s. At the end of this interval the pup was gently detached from the nipple, carried by hand to the opposite end of the alley, and placed facing away from the mother. On reward (R) trials the mother was positioned on her side at the end of the alley and the pup was permitted to attach to a nipple for 15 s. On non-reward (N) trials the mother was removed from the alley and the pup was picked up when it reached the end of the alley. Time to the nearest 0.01 s for the pup to reach the end of the alley on each trial was recorded. In experiment 1 ($n=5$) preliminary training was followed by 5 massed (3-s intertrial interval) R trials, a 15-min retention interval during which the pup was returned to the deprivation chamber, and 25 massed trials in which R and N trials alternated (ALT) in blocks of 5 trials, starting and terminating with 5 R trials. In experiment 2 the initial 5 R trials and the retention interval were not included and two groups ($n=6$ each) were formed: a 5-trial ALT group (as in the last 25 trials of experiment 1), and a group designated continuous reward (CR). Group CR was rewarded on every trial in the acquisition phase by access to a nipple for 15 s. A 10-trial extinction phase began, without signal, immediately after the last acquisition trial. Pups meeting an extinction criterion of 100 s on any trial were terminated and assigned a nominal latency of 100 for all remaining trials.

The results of experiment 1 are shown in Fig. 1. Approach time decreased systematically over the first 5 R trials ($P<0.05$). After the retention interval, 'savings' was reflected in the first 5 R trials of the ALT phase, the block and block-by-trials interaction effects were both significant ($P<0.05$). This was the first clear evidence of which we were aware of systematic acquisition of an instrumental appetitive response and savings in reacquisition in rat pups as young as 10 d of age. There is also a systematic influence on approach times of alternating blocks of R and N trials across the 25 trials after the retention interval: approach times decrease and increase, respectively, in the R and N blocks ($P<0.01$).

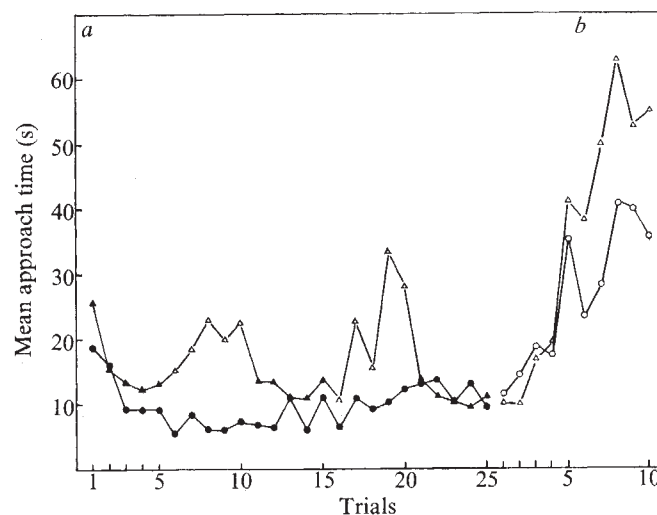


Fig. 2 Mean approach latencies in acquisition (a) and extinction (b) for the two groups in experiment 2. Δ , $\blacktriangle$, ALT; $\bullet$, $\circ$, CR; $\blacktriangle$, $\bullet$, R; Δ , $\circ$, N.

Figure 2 depicts mean approach latencies in acquisition and extinction for the two groups in experiment 2. Acquisition performance of the ALT group again reflected a behavioural sensitivity to 5-trial blocks of R and N trials. No such patterning was apparent in the CR group, where there was systematic reduction in approach time characteristic of learning across acquisition trials. Both groups showed clear extinction of approach behaviour with successive non-rewards ($P < 0.01$). The tendency for the ALT group to extinguish at a faster rate than CR may reflect an extension of the successive 'extinctions' in acquisition.

These results implicate contact with the mother and/or sucking on a dry nipple as sufficient reinforcing events to establish and maintain approach behaviour in 10-d-old rats. Puerperal females under a general anaesthetic, within the range of dosages we used, do not demonstrate normal patterns of milk release from the nipple, and rarely release milk when stimulated by the sucking of a single pup¹⁸. We have not observed the characteristic milk-ingestion reflex in the pup while it was attached to a nipple of an anaesthetised mother, and we did not find an increase in body weight between pretraining and the end of training. It is therefore unlikely that the pups obtained milk during the brief nursing episodes. If the pups did not receive milk, some other feature(s) of the contact-suckling episodes must have served as the reinforcing stimulus. But to attribute reinforcing properties to contact-suckling we must rule out any distance cues—for example, odour and visual—as factors that strengthen and weaken the approach response. Visual cues are ruled out because the eyes of 10-d-old rats are not yet open, but the extent to which rats of this age can use odour cues is not clear. In the presence of a maternal odour, spontaneous activity in 10-d-old rats is reduced¹⁹. Rats as young as 12 d of age, but not younger, prefer shavings from home cages to clean wood shavings, presumably on the basis of odour²⁰. On the other hand, 10-d-old pups given a choice between their own mother and a nulliparous female show virtually no tendency to approach either one; and the lactating female does not begin to emit the maternal pheromone until about 14 d *post partum*, which is the age at which the young are first responsive to the pheromone²¹. In our own data, there is nothing to suggest that odour cues are the basis for differential responding on R and N trials. Odour cues cannot account for the extinction differences between the two groups found in experiment 2 because both groups were run in identical conditions in this phase. These extinction differences may reflect an associative factor which develops differentially in the two groups in acquisition. As a final check on the involvement of odour cues in our experiments, we have replicated the basic finding of experiment 1 (ALT condition) using a procedure in which the anaesthetised mother is present on all trials at the end of the alley. The mother rat is behind a barrier which is raised on R trials after the pup reaches the goal box, but not on N trials. This procedure rules out the possibility that distance cues emanating from the mother on R but not on N trials control the patterned responding in the ALT group.

These results suggest that the development of forebrain structures in the rat during weeks 3 and 4 of life may not be essential for at least certain kinds of learned response suppression. In our experiments appetitive extinction, suppression of approach in the absence of the mother, reflects an 'inhibitory' effect in rats at 10 d of age; in experiments using electric shock, suppression of approach responses in rats shows up much later in development. Although the concept of inhibition has been applied in a variety of contexts, it has always lacked precise definition in psychology²², and psychologists, physiologists and biochemists explain a number of disparate phenomena with the term 'inhibition'. Our results demonstrate that the infant rat can learn to anticipate the absence of a previously

present appetitive event at an age before recognised inhibitory neural centres are said to be operating.

This work was supported by the NSF.

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Received April 26; accepted July 6, 1976.

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Models and statistics for species diversity

INTEREST in the pattern of species abundances in a population takes two forms. On the one hand a study of the full distribution of the relative abundances of species may be sought to give insight into the internal mechanisms of the community; in other cases one or more summary statistics which suitably characterise the population may be required to investigate effects of evolutionary or environmental change. The first approach has led

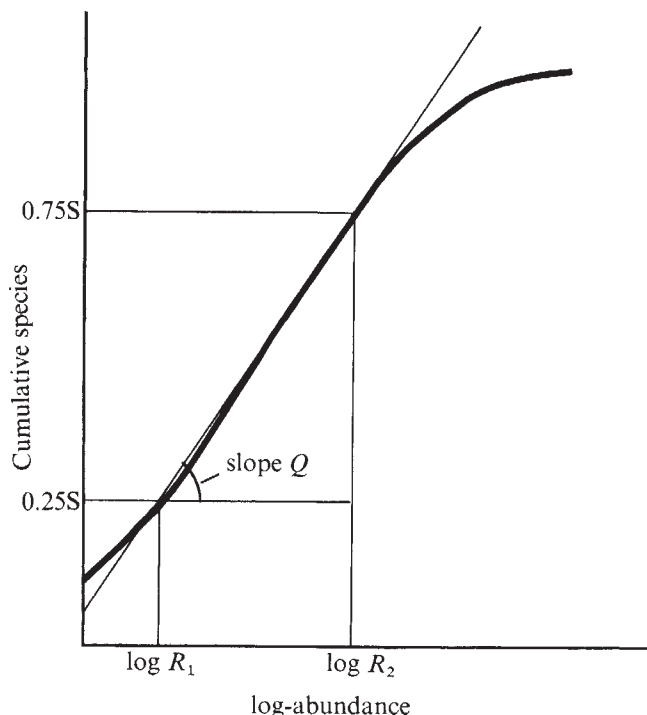


Fig. 1 Evaluation of quantile statistic, Q , from cumulative species abundance curve.

Table 1 Analysis of variance of diversity statistics for 7 replicate samples of moth catches from light traps at 14 sites in the Rothamsted Insect Survey

Source of variation	d.f.	Variance mean square			
		Simpson	<i>H</i>	<i>Q</i>	α
Between sites	13	0.00174	0.977	832.4	1037.5
Within sites	78	0.00014	0.018	6.4	6.4
Between to within sites variance ratio		12.1	54.0	130.5	163.1

Ability of the statistics to discriminate between sites is indicated by the size of the variance ratio, between to within sites.

α , Diversity parameter of the log-series.

to much argument over the appropriate mathematical form for the distribution of abundances, often in the belief that the acceptance of a particular mathematical model will tell us something about the causal biological mechanism. In our opinion, however, the primary purpose of fitting a mathematical model is to smooth the data and enable efficient estimation of population statistics from the parameters of the model; for this we would choose the simplest model which adequately describes the data. Further, there are clear advantages in fitting a single model to describe a series of sets of data—even if this model does not give the best fit to each one—rather than fitting several different models, as has often been suggested, particularly in the literature on succession.

The use of a single diversity statistic to summarise the full

distribution of the relative abundances of species has obvious appeal, in spite of recent criticism of many statistics in common use. Here again we would make no pretence at functional modelling but suggest that species diversity is most usefully regarded as a descriptive statistic for multispecies populations of the same type as, say, population density. Like average density, it is best represented by a statistic that does not over-emphasise the commonest or the rarest species, particularly as species frequency distributions are usually very long tailed. We have strongly criticised¹ the two diversity statistics in common use, the information statistic (*H*) and Simpson's index, because of their sensitivity to the few commonest species, the numbers of which we have found to fluctuate erratically from year to year, rather than to those more stable species with medium abundance.

An alternative diversity index which does not suffer from these disadvantages is based on the slope of the cumulative species curve in the mid-range of abundances (Fig. 1). Mathematically we define

$$Q = S/2 \log(R_2/R_1)$$

where *S* is the total number of species in the sample and *R*₁ and *R*₂ are the lower and upper quartiles of the species abundance distribution (see ref. 2, p. 226). Clearly, *Q* is much less sensitive to the commonest species in the sample, than *H* or Simpson's index, and we have found that it shows smaller relative variability between years and discriminates better between different sites (Table 1).

Fitting a model to the data enables the diversity statistics to be

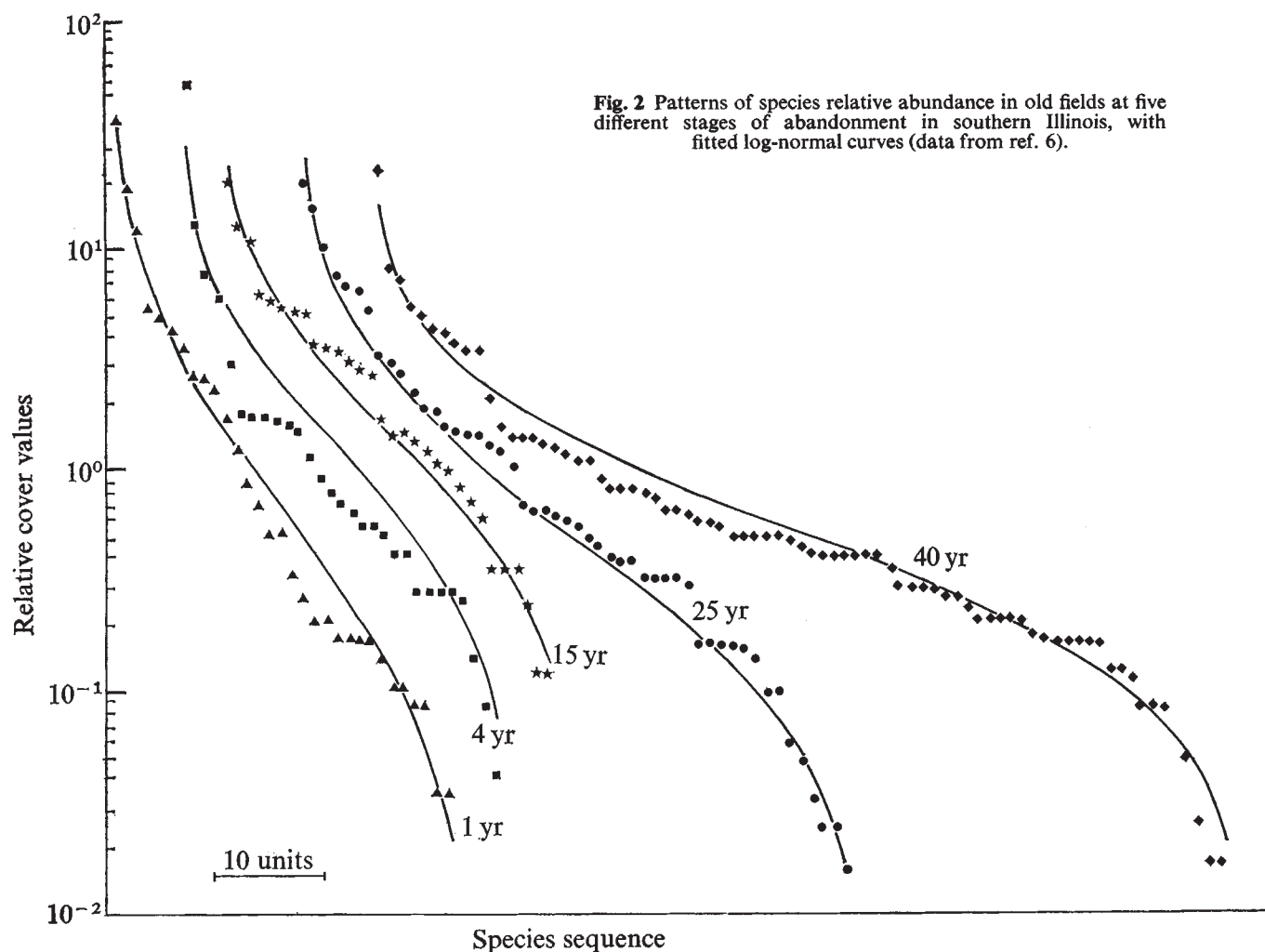
**Fig. 2** Patterns of species relative abundance in old fields at five different stages of abandonment in southern Illinois, with fitted log-normal curves (data from ref. 6).

Table 2 Diatom samples showing replicate variability of the mid-range statistic Q evaluated directly from the data and from the parameters of the log-series and log-normal models (data from ref. 4)

	1	2	3	Replicate 4	5	6	7	8	Mean	Variance
Empirical Q	20.9	17.9	26.4	22.4	20.1	19.2	19.8	23.7	21.3	7.54
Log-series α	19.2	18.3	20.4	19.9	20.5	19.7	18.2	19.9	19.5	0.77
Log-normal cS^*/σ	21.9	20.7	24.3	21.4	23.8	22.7	21.2	23.1	22.4	1.67

estimated much more efficiently than by direct evaluation from the relative frequencies. The mid-range statistic Q is particularly simply represented in terms of the parameters of both the log-series and log-normal models; for the log-series $Q = \alpha$, Fisher *et al.*'s index of diversity³, whereas for the log-normal $Q = cS^*/\sigma$, where S^* is the hypothetical number of species in the population, σ is the standard deviation of logged abundances, and c depends on the proportion of species present in the sample. When all species are present, $S^* = S$ and $Q = 0.371S/\sigma$. Fitting a model enables all the information in the data to be utilised in estimating Q , this should result in smaller variation between replicates. This is shown in Patrick's diatom samples⁴ (Table 2) for which Q has been estimated directly from the data and from the parameters of the fitted log-series and log-normal models, for each of the eight replicates. The mean values of Q are similar for all three methods but the advantage of fitting a model is shown in the resulting smaller replicate variability; this will enable better discrimination between sites (Table 1). The choice of model is of secondary importance but the log-series parameter α shows somewhat smaller variability than the log-normal S^*/σ (Table 2), demonstrating the advantages of using the simpler model, even though the log-series is, for this series of data, nearly always a worse fit⁵.

distribution fitted to all the data we may use the parameters from the model to chart the increase in diversity, described, not by the number of species S , or evenness σ , taken individually but by their ratio S/σ (Table 3), which more closely reflects the visible trend.

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Table 3 Change of diversity with succession in abandoned fields in Southern Illinois (data from ref. 6)

Stage of succession (yr)	1	4	15	25	40
Information statistic, H	2.0	1.7	2.8	2.9	3.2
Mid-range statistic, Q	5.6	8.4	8.0	10.0	24.1
Number of species, S	31	29	30	50	77
Log-normal σ	1.9	1.5	1.3	1.7	1.4
$Q = 0.371S/\sigma$	6.2	7.1	8.4	10.7	20.6

The importance of choice of diversity statistic is also seen in data by Bazzaz⁶ on the change of pattern with plant succession in old fields in Illinois (Fig. 2). In this instance, the pattern of ecological succession, as summarised by May⁷, assumes that in the early stages of succession the Niche Pre-emption Model applies and the ranked abundances of the species may be expected to follow a geometric series. Later, a multiplicity of other factors affecting abundance come into play, and the distribution of species abundance moves closer to the log-normal form. Although this sequence of models may have theoretical appeal, on purely statistical grounds the use of two models seems unjustified, for the data seem to support just as well the use of the same, log-normal distributional model to fit each stage of succession (Fig. 2).

Bazzaz evaluates the information statistic, H , for fields at each stage of succession, and argues that after an initial drop, diversity increases rapidly from year 5 to 15 and then only slowly over the remaining years (Table 3). H seems to reflect the relative abundance of a small number of dominant species. The pattern suggested by the great majority of species is, however, very different, indicating a slow initial increase in diversity followed by more rapid increase in the later stages, changes reflected by the mid-range statistic Q . With one common

Errata

In the article "Promotion and retardation of heat transfer by electric fields" by Y. Asakawa (*Nature*, **261**, 220; 1976) the last sentence to the legend to Fig. 2 should read . . . The cylinder was heated within an electric furnace, and cooling was 1.7 times faster (*b*) and 1.8 times slower (*c*) than in case *a*. and not as printed.

In the article "Age relationships along the Hermitage Bay-Dover Fault System, Newfoundland by J. Blenkinsop, P. F. Cucman and K. Bell (*Nature*, **262**, 377; 1976) the figure in the fourth line of the first paragraph should be 400 Myr and not 4 Myr as printed.

In the article by O. K. Wilby (*Nature*, **262**, 387; 1976) the title should read ". . . and the effects of antibiotics . . ." and not antibodies as printed.

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matters arising

Nuclear composition of cosmic rays

WHEN the phenomenological constant in the local source model of Rasmussen and Peters¹ is given a more physical basis, its large value cannot be easily understood.

I begin by deriving their equation (1). For each species i , the steady-state equation for the density η_i^b [cm⁻³ GeV⁻¹], of high energy particles coming from a background of uniformly distributed sources with strength S_i^b [cm⁻³ s⁻¹ GeV⁻¹] and undergoing spallation is

$$\frac{d\eta_i^b}{dt} = 0 = -n\sigma_i c\eta_i^b + S_i^b + \sum_{j>i} n\omega_{ij} c\eta_j^b \quad (1)$$

where n [cm⁻³] is the mean density of hydrogen, c is the velocity of light, and the cross sections σ_i and ω_{ij} [cm²] were defined by Rasmussen and Peters. To write equation (1) in terms of the total density, η_i , and total source strength, S_i , let L_i [s⁻¹ GeV⁻¹] be the mean luminosity of the cosmic ray sources, then

$$\eta_i^b = \eta_i - \eta_i^l, S_i = NL_i/V_G \quad (2)$$

$$S_i^b \sim (1 - N^l/N)S_i \quad (3)$$

$$\eta_i^l = \sum_a \frac{L_i^a f_a}{4\pi c d_a^2} = \left(\sum_a \frac{L_i^a f_a}{4\pi c d_a^2} \frac{V_G}{NL_i} \right) S_i = \frac{KS_i}{nc} \quad (4)$$

where N is the total number of sources, V_G is the containment volume, L_i^a is the luminosity of and d_a the distance to the a th of the N^l local sources, and f_a is the enhancement from diffusion. Note that equation (4) pertains to stable nuclei and assumes negligible spallation. After substituting equations (2)–(4), equation (1) becomes

$$\frac{S_i}{n} = \frac{\sigma_i c \eta_i - \sum_{j>i} \omega_{ij} [c\eta_j - KS_j/n]}{1 + \sigma_i K} \quad (5)$$

(if $N^l/N \ll 1$). If $S_i/n \rightarrow S_i$ (the relative strength) and $\eta_i c \rightarrow F_i$, then equation (5)

becomes equation (1) of Rasmussen and Peters.

Next, approximate the transport from a steady source by assuming isotropic, three-dimensional diffusion in an infinite medium (see ref. 2). Then the equilibrium value of the factor f_a is given by³

$$f_a = (d_a c/D) \quad (6)$$

and, after an interval Δt , the root mean square distance, r_{rms} , for newly produced particles is

$$r_{rms} = (6D\Delta t)^{1/2} \quad (7)$$

where $D(= \lambda c/3)$ is the diffusion constant and λ is the scattering mean free path. If spallation is negligible, the random path $c\Delta t$ must be much smaller than a spallation mean free path for the heaviest element injected by a source. Thus equation (7) implies that the boundary of the local source region, R^l , is

$$R^l = (2\lambda \xi / n \sigma_v)^{1/2} = 180(\lambda/n)^{1/2} \text{ pc} \quad (8)$$

where $\xi = 0.1$ permits up to 10% spallation, $\sigma_v (= 2 \text{ barn})$ is the spallation cross section for uranium, and where the units for λ and n are pc and cm⁻³. Also, if all of the sources are distributed uniformly, the mean number of local sources is just

$$\langle N^l \rangle = \left(\frac{4\pi(R^l)^3}{3V_G} \right) N = 7.2 \times 10^{-5} (\lambda/n)^{3/2} N \quad (9)$$

for $V_G = 10^{67} \text{ cm}^3$.

Rasmussen and Peters then found the source abundances of Li, Be, and B were minimised if $K = 0.02 \text{ mbarn}^{-1}$. From equations (4), (6), and (9) this means

$$d_* = 0.90(N^l/\langle N^l \rangle)^{1/2} (\lambda/n)^{1/2} \text{ pc} \quad (10)$$

where d_* is the equivalent distance to the local sources defined by

$$\frac{1}{4\pi d_*} = \frac{1}{N^l} \sum \frac{L_i^a/L_i}{4\pi d_a} \quad (11)$$

Note that if the local sources were identical and spherically distributed, then equation (11) says $d_* = (2/3)R^l(L_i/L_i^a)$.

While if there is only one local source $d_* = d_a(L_i/L_i^a)$.

Equation (10) poses a severe problem for the local source model. If $N^l \approx \langle N^l \rangle$, $d_* \sim 1 \text{ pc}$, which implies either the source(s) is extremely close or extremely luminous. If $N^l \sim 30\langle N^l \rangle$, then d_* becomes acceptable, but the total number of sources becomes uncomfortably small. Using equation (9), such a fluctuation yields 2,300 sources (5 local) but with a probability of 9.2×10^{-7} (according to Poisson statistics). These problems become even more severe if the enhancement factor, f_a is set to unity.

To summarise, the size of the phenomenological constant K seems inconsistent with a physical interpretation in terms of source properties and distances. The difficulties in understanding the small value of d_* arise because the model requires the local source(s) to contribute extremely large amounts of unspalled C and O to match the observed L/M ratio.

I thank C. McKee and R. Gordon for discussions. This work was supported by NASA.

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¹ Rasmussen, I. L., and Peters, B., *Nature*, **258**, 412 (1975).

² Lingelfelter, R. E., *Nature*, **224**, 1182 (1969).

³ Ginzburg, V. L., and Syrovatskii, S. I., *The Origin of Cosmic Rays*, 301 (MacMillan, New York, 1964).

The cosmic X-ray background

MATTER-antimatter annihilation has had great appeal in astrophysical situations involving apparently overdrawn energy budgets. In spite of observational evidence that antimatter is no panacea¹, annihilation theories rise periodically, phoenix-like, in explanation of a wide class of astrophysical phenomena. In a recent letter, Carlqvist and Laurent² proposed that the X-ray background (1–100 keV) arises from Compton scattering of starlight photons by annihilation-produced, relativistic electrons and positrons. We show here that the Carlqvist-Laurent proposal is in conflict with γ -ray and neutrino observations. Were the X-ray background caused by annihilation produced electrons, the 100-MeV γ -ray flux would exceed that observed³ by six to ten orders of magnitude, the muon-neutrino flux would exceed the observational upper limit⁴ by one to five orders

of magnitude and, a flux of 511 keV annihilation γ rays would be produced in excess of that observed⁵ by up to eight orders of magnitude.

To produce the cosmic X-ray background, Carlqvist and Laurent² require the Universe to be filled with annihilation-produced electrons and positrons at a density $n_e \approx 10^{-9} \text{ cm}^{-3}$. Since, in a typical annihilation, roughly one γ ray ($\sim 100 \text{ MeV}$) is produced for each electron¹, the γ -ray flux at Earth should be

$$F_\gamma \approx cn_\gamma \approx cn_e \approx 30 \text{ cm}^{-2} \text{ s}^{-1} \quad (1)$$

This predicted flux exceeds that observed³ by some six orders of magnitude. To avoid this embarrassment, Carlqvist and Laurent² suggest that the γ rays may be absorbed if annihilation were to occur in galactic nuclei instead of intergalactic space. Since the range of a 100-MeV photon exceeds the range of a 100-MeV electron (because of annihilation, the range of a 100-MeV positron is even shorter), it is impossible to absorb the γ rays without, first, stopping the electrons. Furthermore, since the electrons are tied to magnetic fields², they must pass through more material than the γ rays to escape from the annihilation volume. There is no way to bury the γ rays without also interring the electrons and positrons.

Although the previous discussion shows that the density of annihilation-produced electrons must be at least six orders of magnitude lower than that required by Carlqvist and Laurent² to produce the X-ray background, it is of interest to consider two other, independent constraints which support this conclusion.

As Steigman and Strittmatter⁶ have shown, a typical annihilation yields two detectable μ neutrinos for every three electrons. Such annihilation-produced neutrinos are extremely difficult to detect and have been of only marginal value in constraining matter-antimatter theories⁶. The Carlqvist-Laurent proposal² is virtually unique in predicting a flux of μ neutrinos which exceed the upper limit to the observed flux⁴ ($F_{\nu_\mu}(\text{obs}) \lesssim 3 \text{ cm}^{-2} \text{ s}^{-1}$).

$$F_{\nu_\mu} \approx cn_{\nu_\mu} \approx 2/3 cn_e \approx 20 \text{ cm}^{-2} \text{ s}^{-1} \quad (2)$$

The neutrinos, of course, will not be absorbed in the annihilation region.

Finally, a relativistic positron in a medium of density n will annihilate in a time $t \approx 10^{14} n^{-1} \text{ s}$, producing two 511-keV γ rays. The predicted flux of these annihilation γ rays is

$$F_{511} \approx 10^6 n \text{ cm}^{-2} \text{ s}^{-1} \quad (3)$$

For an average intergalactic density⁷ ($n \approx 10^{-7} \text{ cm}^{-3}$), the predicted flux exceeds that observed⁵ ($F_{511}(\text{obs}) \lesssim 2 \times 10^{-3} \text{ cm}^{-2} \text{ s}^{-1}$). In the Carlqvist-

Matters arising

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Laurent scheme², the situation is much worse, however. They require the annihilation to occur in quasars or galactic nuclei where the ambient density is very high. For example, for an average galactic density ($n \approx 1 \text{ cm}^{-3}$), the 511-keV flux would exceed that observed⁵ by some eight orders of magnitude. Furthermore, every electron or positron which annihilates to produce a 511-keV γ ray was originally produced along with γ rays and neutrinos. Thus, the γ -ray flux would exceed that observed by some ten orders of magnitude and the μ -neutrino flux would exceed the observational upper limit by some five orders of magnitude! (The reason for the increase over our earlier estimates is that Carlqvist and Laurent² assumed that the electrons and positrons lived for $\sim 10^{10} \text{ yr}$; however, because of annihilation, their lifetimes are much shorter and, thus the original annihilation rate must be increased by the ratio of the age of the Universe to the lifetime of the electrons.)

It is clear that observational limits to the flux of annihilation products (γ rays and neutrinos) provide serious constraints to annihilation as an astrophysical energy source^{1,6}. Since the recently proposed² annihilation explanation of the X-ray background is incompatible with these limits by many orders of magnitude, it is untenable.

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¹ Steigman, G., *Nature*, **224**, 447 (1969); *Cargese Lect. in Phys.*, **6**, 505 (1973); *Proc. IAU Symp.*, **63**, 347 (1974); *A. Rev. Astr. Astrophys.* (in the press).

² Carlqvist, P., and Laurent, B., *Nature*, **260**, 225 (1976).

³ Fichtel, C. E., et al., *Astrophys. J.*, **198**, 163 (1975).

⁴ Wolfendale, A. W., and Young, E. C. M., *CERN Neutrino Mtg Proc.*, 95 (1969).

⁵ Johnson, W. N., III, et al., *Astrophys. J. Lett.*, **172**, L1 (1972).

⁶ Steigman, G., and Strittmatter, P. A., *Astr. Astrophys.*, **11**, 279 (1971).

⁷ Gott, J. R., III, et al., *Astrophys. J.*, **194**, 543 (1974).

CARLQVIST AND LAURENT REPLY—In Steigman's¹ criticism of our paper² he raises three points, with which we shall deal in order. One of these points con-

cerns the γ rays produced by collisions of annihilation electrons (positrons) with positrons (electrons) in intergalactic space. Steigman believes that two 511-keV γ rays are formed in each electron-positron annihilation. This is not correct. If one highly relativistic electron (positron) annihilates on a positron (electron) at rest, one very high energy photon is produced and one photon with an energy in the region $m_e c^2/2$ to $\sim m_e c^2$. Consequently no discrete γ line is formed but a continuous spectrum. Steigman further uses a value of 10^{-7} cm^{-3} as the average intergalactic particle density, which is rather the density of smeared out galactic material. The true intergalactic density is not known. It should be pointed out that there is no reason why electrons and positrons must penetrate galaxies, which may be shielded by their magnetic fields.

A second point that Steigman raises concerns the production of γ rays in connection with the proton-antiproton annihilation. His objection here is based on a homogeneous model and does not apply to the model used by us. If annihilation occurs as a result of collisions between matter and antimatter objects which are sufficiently dense, most of the γ rays from the annihilation will be absorbed by the objects themselves^{3,4}. The annihilation electrons and positrons may, on the other hand, be temporarily confined by magnetic fields to regions with a much lower density than in the surroundings and then be released into intergalactic space. One cannot therefore state categorically, as Steigman does, that the γ rays cannot be absorbed without stopping the electrons and positrons.

Steigman's third point concerns μ neutrinos formed on annihilation. He has used our suggestion to estimate the flux, and finds that it exceeds the measured number by one order of magnitude. It should be stressed that this estimate is necessarily very uncertain for two reasons. First, we have calculated the electron density only to an order of magnitude; and secondly, it is questionable how much value one should attribute to the old experiments referred to.

Finally it should be pointed out that Steigman has implicitly assumed an homogeneous cosmological model. If, however, the metagalactic model⁵⁻⁷ is adopted, neutrinos and γ rays created long ago would have escaped through the boundary of the system.

In itself it is legitimate to use specific models but Steigman uses specific models in an illegitimate way to draw general conclusions.

¹ Steigman, G., *Nature*, **262**, 821-822 (1976).

² Carlqvist, P., and Laurent, B., *Nature*, **260**, 225-226 (1976).

³ Alfvén, H., and Elvius, A., *Science*, **164**, 911-917 (1969).

⁴ Elvius, A., *IAU Symp.*, **44**, 306-310 (1972).

⁵ Alfvén, H., and Klein, O., *Ark. Fys.*, **23**, 187-194 (1963).

⁶ Klein, O., *Nature*, **211**, 1337-1341 (1966).

⁷ Alfvén, H., *Phys. Today*, **24**, 28-33 (1971).

reviews

Games of chance and necessity

I. Prigogine

Das Spiel: Naturgesetze Steuern den Zufall. By Manfred Eigen and Ruthild Winkler. Pp. 404. (R. Piper: Munchen and Zurich, 1975.) DM38.

TODAY, we are very far from the ideals of classical science centred on stability and permanence. We now see that such qualifications apply only to very limited aspects. Wherever we look, we discover evolutionary trends leading to diversification and increasing complexity. The monograph by Eigen and Winkler provides us with an excellent introduction to this new, more dynamic, vision of the physical world.

Dr Eigen is one of the key figures in contemporary physical chemistry and molecular biology, but the present work goes far beyond the field to which Eigen has made important contributions. It spans an extraordinarily wide range from fundamental concepts in mathematics and theoretical physics such as game theory or symmetry, to problems of aesthetics and epistemology. It shows that a synthetic approach to both the scientific and cultural problems of our time is still possible.

The central theme is self-organisation. How can complexity arise spontaneously? The approach taken by the authors is clearly indicated in their Preface: "... All happenings in our world are similar to a big game in which only the rules are fixed *a priori* . . . We consider the game to be the natural phenomenon which, with its dichotomy of chance and necessity, is the basis of all change" (translation by the reviewer). This starting point leads to the search for unity in nature "as revealed by relationships between structures" and for unity between nature and spirit "as revealed by the existence of languages both in molecular biology and in human communication". The central theme, Nature as a game, is analysed using many examples.

It is impossible to analyse in detail the content of this monograph, which is subdivided into four parts, covering the simplest situations which game theory and physics can analyse, and ranging to problems of ecology, linguistics and aesthetics (the chapter on music is specially informative and original).

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The concept of game (or play) is taken as the basis of both the unity and the self-organisation of the physical world. This theme is a recurrent one in the German *Natur Philosophie* of the romantic period (*Histoire de la Philosophie*, III, Encyclopedie de la pléiade, Gallimard, 1974). According to this view, game (or play) is the very expression of the creativity of Nature. It is interesting to note the contrast between the intellectual atmosphere of Eigen and Winkler's book with that of Monod's classic *Chance and Necessity* (Editions du Seuil, 1970). Monod stresses the analogy between living beings and chemical machines, and this analogy is repeated several times. Monod is, in fact, following in this respect the mechanistic cartesian tradition so strong in French thought. It is not surprising that, starting from this view, he has great difficulties in incorporating adaptation and biological evolution in his theory. Life appears as a phenomenon existing *de facto* but not *de jure*, as its presence can only be understood in terms of extremely unlikely, even unique, events.

Eigen and Winkler avoid, from the beginning, such difficulties as games having both deterministic and stoch-

astic aspects: the rules are given in a deterministic fashion, whereas the time sequence of events contains stochastic elements. Instead of opposing chance and necessity, Eigen and Winkler show how these two aspects may cooperate to produce the complexity and diversity we observe in Nature.

Obviously, in a book devoted to a synthetic view of Nature the problems are of such a wide range and depth that different opinions may be held. This is only one more positive aspect which must be credited to the authors.

For example, it is difficult to accept the postulate of Eigen and Winkler that long time processes may all be identified with games. Games are mathematically associated with semi-groups, such as those involved in Markov processes. On the other hand, classical and quantum mechanics lead to dynamic groups, involving unitary transformation. The transition from dynamic groups to games contains all the difficulties of going from dynamics to the theory of probabilities. There is no reason to believe that the description of the motion of a harmonic oscillator will ever need new elements over and above those contained in dynamics. Inversely, there is no reason

to believe that probabilistic laws such as those describing Mendel's laws will ever be reduced to deterministic schemes. We now begin to understand that a minimum amount of complexity will lead to the introduction of stochastic elements where long time scales are involved (J. Moser, *Stable and Random Motions in Dynamical Systems*, Princeton University Press, Princeton, 1974). It seems that no reduction of Nature to a single fundamental level (either law or game) is possible. We have to accept a more complex situation involving the continued interplay between multiple levels (such as the interplay between macroscopic and microscopic levels emphasised by Bohr and Rosenfeld).

Also, the distinction between rules and events as used by Eigen and Winkler seems to be somewhat too rigid. In particular, when dealing with problems involving human behaviour, we cannot really speak of *given* rules. The games are so highly non-linear that essentially the rules themselves become situation-dependent. A well-known example is that of traffic flow, in which the strategy of the drivers depends on the specific situation (I. Prigogine and R. Herman, *Kinetic*

Theory of Vehicular Traffic, American Elsevier, New York, 1971).

An important feature of the book by Eigen and Winkler comes from the fact that it shows how to go beyond the classic paradigms of molecular biology. Molecular biology has essentially been aiming to identify and isolate the characteristic biomolecules. Because of the very success of molecular biology it becomes today possible to proceed in new directions, and to relate structure on the super-molecular level to the characteristic properties and interactions of these biomolecules. To do so we need as emphasised by Eigen and Winkler, both conservative and dissipative structures. Conservative structures arise mainly through short range interactions (valency and van der Waals' forces), whereas dissipative structures result from chemical kinetics and transport processes if suitable conditions are satisfied. An essential feature of dissipative structure is that they depend on the system as a whole, or alternatively that they are similar to long range forces that lead to coherent behaviour.

Interesting chapters are devoted to the laws of population dynamics and

to Darwinian evolution theory. Again, many points deserve a more detailed discussion. Can Darwinian theory really reduce to a model of molecular kinetics involving competition, similar to that operating for macromolecules? It seems to me that the position of Eigen and Winkler is somewhat optimistic, since the molecular mechanism of genetic fluctuations on one side and the laws of ecology on the other are still far from being clarified.

Still, it is interesting to apply models to specific situations, as in Eigen's original work, to be able to attach a precise meaning to concepts such as the survival of the fittest.

There is a deep concern with human fate. To quote Eigen and Winkler: "Man is neither a mistake of Nature nor is Nature taking care automatically of his preservation. Mankind is participating in a big game the result of which is still open. Man has to develop his potentialities to assert his role as a player in order not to be reduced to a mere object of chance" (translation by the reviewer). □

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Lay guide to microbiology

Invisible Allies: Microbes and Man's Future. By Bernard Dixon. Pp. 251. (Temple Smith: London, May 1976.) £4

It was difficult for me, now approaching the end of a career in microbiology, to judge a book intended for lay-people. Too often 'nit picking' intervened. The opinion which follows is partly my own and partly that of friends and relations, all lay-people (except for an acquaintance with me). The title of the book underlies an admirable intention. That is, it seeks to dispel the attitude, still too common even in our new students, that sees microbiology in terms of medicine and thus all microbes as enemies. How disappointing, for instance, was the BBC Television series *Microbes and Man* in this respect: Dixon's book might well form the basis of a remedial programme. The treatment has less detailed biology than that out of the classic *Microbes by the Million* and is possibly less professional than John Postgate's *Microbes and Man* (obligatory for our first-year students). Dixon's book should be obligatory to all lay people with a spark of interest in their own relationship to the living

world. After a brief, rather sketchy description of microbes, it goes on to describe their role in the biological production cycle, and their exploitation. Besides having value in their own right microbes now play an important role in experimental biology; this, and especially the advances in molecular biology, are well treated but some of the examples used in talking about evolution could have been more relevant. Microbial infection, so often seen as an all-or-none process, is placed in the perspective of ecology. Aspects of microbiology now causing alarm such as germ warfare and genetic engineering are briefly discussed. A general criticism was of the rather concentrated writing and the lack of good diagrams or other illustrations. These undoubtedly would have improved the book considerably and reduced the concentrated wordiness, provided time for reflective pauses and given some indication of the scale of industrial process.

If a good book is one that helps a change of mind then this is undoubtedly a good book. My copy will be to hand to press on those who wonder how I have spent my time and their taxes.

D. E. Hughes

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Ultrastructural masterpiece

Ultrastructure and The Biology of Plant Cells. Pp.312 (49 plates). £27.50. *Plant Cell Biology: An Ultrastructural Approach.* Pp.280. £2.75 paper. By Brian E. S. Gunning and Martin W. Steer. (Arnold: London, 1975.)

THERE is currently available a number of books dealing with the ultrastructure of plant cells, each of which provide a series of excellent electron micrographs. They are, however, often flawed by an indifferent text. The pair of books presented here are a welcome exception. The smaller student book is derived from the other (a reference tome), and they collectively represent a masterpiece. The authors have been wholly successful in the enormously difficult task of illustrating the dynamic nature of cell structure using the essentially static medium of electron microscope snapshots.

The major book, although hideously expensive, is beautifully produced; and the photographs, collected together at the end, are superb. There are 49 plates, almost all of which comprise multiple montages; and virtually every conceivable aspect of plant cell struc-

ture, from general features common to all cells, to specialised structures, such as cuticles, pollen grains and glands are represented. The illustrated material is amply supported by an extensive and erudite text which deals with the subject at a very high level—not really suitable for students—but of great value to university teachers and research workers.

The book begins, as is almost obligatory with this topic, by describing in detail the various methods used to prepare, section and visualise plant material, giving due emphasis to the advantages and shortcomings of all the techniques. After a general chapter on cell structure with particular reference to membranes, the coverage subsequently works from the outside inwards—that is, cell wall and plasma membrane, vacuole and tonoplast, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, plastids, microbodies and sphaerosomes, microtubules and microfilaments, and cell division. As may be expected from these authors, the chapter on plastids is particularly rewarding, with a masterly coverage of the structure of the granum and the prolamellar body. This chapter, however, is merely a prominent peak in a high mountain range. I was particularly impressed by

the careful attention of the authors to comparative sizes of cell constituents. The book is finished off with a scholarly treatment of the cell as an integrated unit and a very comprehensive list of references.

The smaller book is in reality a reprint of the 49 plates of the larger book together with the figure legends and a minimum of extra text. As a book for students it is likely to be quite useful, although it could have been improved by the addition of a précis of the text from the larger book. It seems rather remarkable that the figures of the larger book can be put on sale for one-tenth of the price of the whole book, when we are always being told that half-tone reproduction is extremely expensive. Publishing houses clearly move in a mysterious way . . .

Apart from the price, the larger book is an excellent buy and lecturers might well consider using it as a basic text, recommending their students to use the cheaper version with the photographs.

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Optical multilayers

Optics of Thin Films. (An Optical Multilayer Theory.) By Zdenek Knittel. (Wiley Series in Pure and Applied Optics.) Pp. 548. (Wiley-Interscience: London and New York, February 1976.) £15; \$33.

THE appearance of this volume is timely. Since the appearance of the first book on this subject, in 1955, considerable developments have occurred, and the optical multilayer has become a normal—and in some cases crucial—component in many optical systems. With the availability of computers, multilayer calculations have become straight-forward and great progress has been made in automatic design routines in which the computer is given the desired reflection-transmission curve and is asked to determine appropriate layer thicknesses. Dr Knittel's long experience in the optical industry has put him in a strong position to write with authority on the many developments of the past twenty years. Many of the methods described enable an insight to be obtained on the way in which multilayer properties are influenced by changes in particular layers. Such methods can assist in design problems without the need for mammoth

number-crunching powers and can serve for many simple systems. A useful section is included on inhomogeneous layers which still remain to be fully exploited in the practical field.

The book will form a useful reference work for the multilayer designer. There are one or two unfortunate features which make for difficulties in reading. In the text, the boundaries between layers are envisaged as being in a vertical plane, so that waves are described as going towards right or left, whereas in figures the boundaries are shown as horizontal planes. Some graphs have no labels on the axes and in some cases too little information is given to understand readily the significance of graphs bearing several curves. In one example, curves are given for an anti-reflecting system embedded in a medium of refractive index 1.2—a curious choice in view of the absence of any real material with this value of refractive index. These are, however, fairly minor points.

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The appointment is based in Basingstoke. The salary will be competitive and a company car will be provided. Applications (which will be treated in strictest confidence) to:

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obituary

Harry Nyquist, widely known for his theoretical studies and practical inventions in the field of communications, died on April 4 at the age of 87 in Harlingen, Texas. Born in Nilsby, Sweden, on February 7, 1889, Nyquist was the son of Lars and Katarina Nykvist. He emigrated to Minnesota in 1907 and taught at the Southern Minnesota Normal School in Austin, Minnesota. Nyquist earned his BA, BS and MS degrees from the University of North Dakota, and in the summers worked at the National Bureau of Standards and the US Patent Office.

He joined the American Telephone and Telegraph Company in 1917, shortly after he had obtained his PhD in physics from Yale University, and moved to the Bell Telephone Laboratories in 1934.

From 1917 to 1921 he worked primarily on the development of the metallic telegraph system and methods of measuring telegraph distortion. From 1921 to 1935 he devoted considerable time investigating television techniques, and worked on projects aimed at extending and improving long-distance telephone circuits.

After 1935, Nyquist devoted most of his time to research work where he applied his broad knowledge and analytical ability to the most difficult of challenges in the field of communications development. For the two years before his retirement in 1954 he was Bell Laboratories' Assistant Director of Systems Studies.

Nyquist was awarded 138 patents during his 37 years with the Bell System. Many of his inventions and theories are widely accepted as fundamental to voice, picture and data transmission. His discovery of the conditions necessary to keep feedback circuits stable, the 'Nyquist Criterion', brought him widespread acclaim. It is

used not only in the study of electronic devices, such as amplifiers, but even in the study of human regulating processes—for example, to describe the way in which a person steers a car.

He gave the first quantitative explanation of thermal noise, and through theoretical analysis determined the minimum band of frequencies required to transmit various kinds of communication signals. These studies laid the foundation for modern information theory and data transmission. In the television field, Nyquist invented a method of transmission used in broadcasting today, and also discovered a way to correct delay distortion of TV images.

Among the many honours Nyquist received were: the Mervin J. Kelly Award of the AIEE (1961), the National Academy of Engineering founders Medal (1969) and the Rufus Oldenburger Medal from the ASME (1975).

Harald Trap Friis, a pioneer in the development of radio communications and an initiator of microwave radio transmission and radio astronomy, died on June 15. He was 83 years old.

Friis was born in Naestved, Denmark, February 22, 1893. He attended the Royal Technical College in Copenhagen, where he received his Electrical Engineering degree in 1916 and his Doctorate in Science in 1938. From 1917 to 1918 he worked as Technical Advisor at the Royal Gun Factory in Copenhagen. In 1919, upon receipt of a Fellowship from the American-Scandinavian Foundation, he moved to the United States to study at Columbia University.

In 1920 Friis joined the Western Electric Company's research department, the predecessor organisation to Bell Laboratories, and in the twenties

he designed the first commercial 'double - detection super - heterodyne broadcast radio receiver', the forerunner of present-day radios. In 1923, Friis spent several months in England devising receivers for a long-wave transatlantic telephone link. In the decade that followed he is credited with designing a receiver that automatically compensated for fading signals, an improved directional antenna, and methods for recording static and measuring fading shortwave signals.

His most famous achievement was, however, in the design of antennae. One designed by Friis together with Bell Labs colleague, Karl Jansky, was the first to detect 'star noise' in 1932—the birth of radio astronomy. The rhombic antenna designed by Friis and another colleague, Edmond Bruce, found worldwide use in shortwave radio telephony, and yet another of his antennae—the Multiple Unit Steerable Antenna—made it possible, Friis said, "to unravel the phenomena of short-wave transmissions". He was the co-author of the book *Antennas: Theory and Practice* (1952).

In 1938 Friis moved from shortwaves to microwaves and created the horn-reflector antenna, now seen on tower tops all over the country. During this period Friis put together the basic elements of a microwave telephone system. A year after the end of the war, the first experimental microwave circuits based on Friis' designs were installed for domestic use and today, Bell System microwave radio facilities provide some 380 million miles of long distance telephone circuits.

Following his retirement, Friis spent 10 years as a research consultant to the Hewlett-Packard company in Palo Alto, California. He published an autobiography, *Seventy Five Years in an Exciting World* in 1971.

announcements

Meetings

September 9–10, **565th Meeting of the Biochemical Society**, Stirling (The Meetings Officer, The Biochemical Society, 7 Warwick Court, Holborn, London WC1R 5DP).

September 15–17, **Herbicides and Fungicides, Factors Affecting Their Activity**, Bangor, Wales (Dr S. Turner,

Reckitt and Colman Pharmaceutical Division, Dansom Lane, Hull HU8 7DS).

October 11–13, **Transportation and Communications**, Genoa (Segretaria Generale IIC, Villa Piaggio, Via Pertinace-16125 Genova, Italy).

October 22, **Human and Animal Endoparasites**, London (The Assistant Secretary, Society of Chemical Industry,

14 Belgrave Square, London SW1X 8PS).

November 15–26, **Weather Forecasting in Africa**, Dakar, Senegal (The Secretariat, WMO, Geneva, Switzerland).

November 19, **The Newer Applications of Pyrethroids**, London (The Assistant Secretary, Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS).